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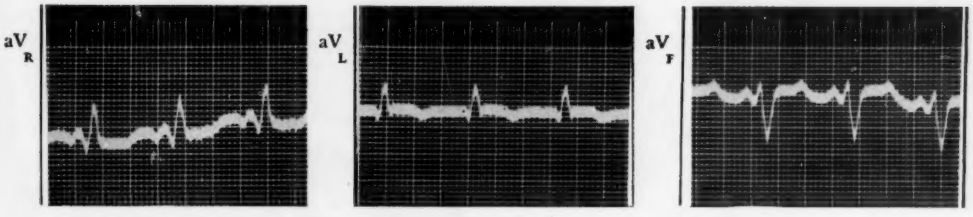

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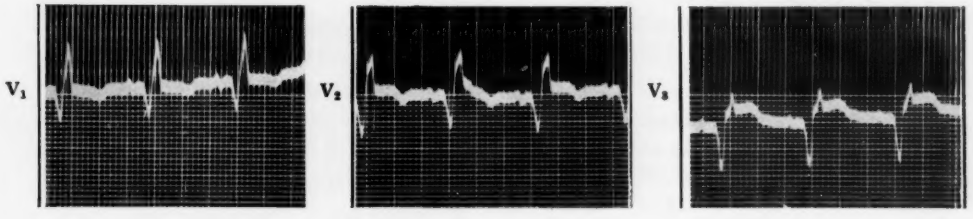

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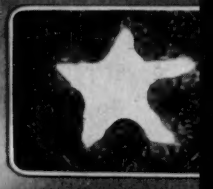

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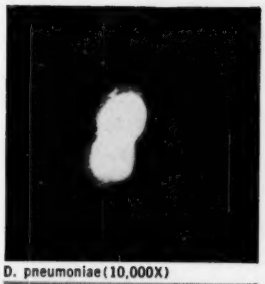

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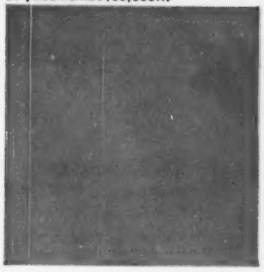

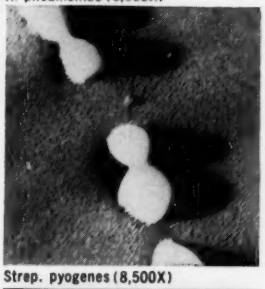

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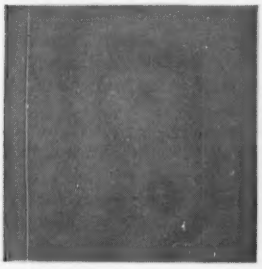

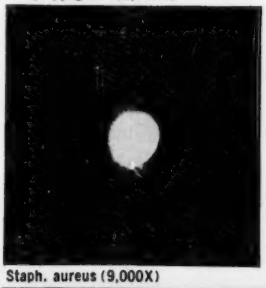

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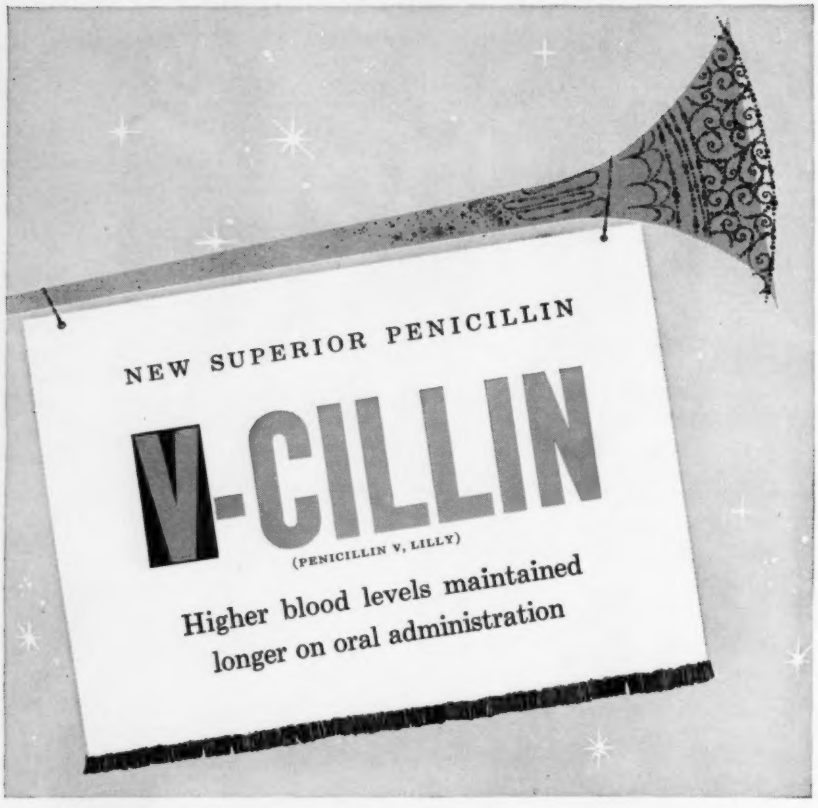

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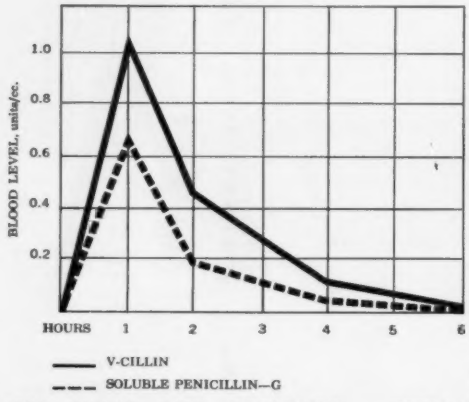

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THE EFFECTS OF VENOUS CONGESTION OF THE LIMBS AND PHLEBOTOMY UPON RENAL CLEARANCES AND THE EXCRETION OF WATER AND SALT. II. STUDIES IN PATIENTS WITH CONGESTIVE FAILURE[1]

By WALTER E. JUDSON, J. D. HATCHER,[2] WILLIAM HOLLANDER, MEYER H. HALPERIN, AND ROBERT W. WILKINS

(From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.)

(Submitted for publication April 22, 1955; accepted July 6, 1955)

A previous report from this laboratory (1) has shown that venous congestion of the limbs in normal subjects and in hypertensive patients before and after splanchnicectomy causes a decrease in urine flow and sodium excretion. In the present study these observations were extended to include also the renal functional effects of phlebotomy (as well as of venous congestion) in patients with congestive heart failure. In addition, cardiohemodynamic measurements were made simultaneously in a majority of patients in order to determine whether changes of cardiac output, or blood pressure in the pulmonary artery, or right ventricle are consistently related to the renal excretory responses. The details of the cardiohemodynamic studies have been presented in a separate communication (2).

PATIENTS

All of the twenty-one patients were considered to be in congestive heart failure on the basis of their clinical cardiovascular symptoms and signs and their abnormal hemodynamic responses to exercise. Seven patients had rheumatic heart disease, six arteriosclerotic heart disease, three hypertensive cardiovascular disease, and one luetic heart disease. Two patients (W. D., W. H.) had cor pulmonale with very severe congestive failure. Two other patients (D. K., C. Di.) had elevated pulmonary arterial pressures without electrocardiographic or roentgenographic evidence of right ventricular enlargement, but the hemodynamic measurements of right ventricular filling pressure during exercise indicated mild right ventricular failure. All of the patients, except D. K., H. A., and R. H., had moderate to severe peripheral edema during the study. All of the patients were on maintenance oral digitalis therapy, but none of them had received mercurial diuretics for at least one week prior to the procedure. Although some restriction of the amount of salt in the diet had been prescribed, most of the patients had an intake of more than 3 gm. of sodium chloride a day.

PROCEDURE AND METHODS

The patients were studied in the post-absorptive state without sedative pre-medication. An indwelling catheter was placed in the urinary bladder. After standard priming doses, a sustaining infusion containing inulin (3.3 per cent) and para-aminohippurate (0.5 per cent) in 3.5 per cent glucose and water was started and given at the rate of 1.2 ml. per minute by means of a calibrated drip. A double lumen intracardiac catheter was inserted into the right heart so that simultaneous records of the pulmonary arterial and right ventricular pressures could be obtained. An indwelling needle was maintained in the brachial artery.

During the control period, three to four urine collections were usually obtained. Urine was collected at the end of each 10 to 15-minute period by washing out the bladder with 20 ml. of distilled water and evacuating the residual urine by displacement with air. Every twenty minutes arterial blood samples were obtained for the determinations of inulin, para-aminohippuric acid, chloride, sodium, potassium, and hematocrit. During the control period, one or two cardiac output determinations were made by the direct Fick method as well as measurements of the central vascular and peripheral arterial pressures as previously described (2). In 15 patients, after the control measurements had been obtained, cuffs on the thighs were inflated at slightly less than diastolic pressure (usually 70 mm. Hg). Venous congestion was maintained for 33 to 71 (average 55) minutes, during which time collections of urine were made at 10 to 20-minute intervals. In six other patients, venesections of 450 to 700 milliliters were accomplished over a period of 10 to 40 minutes. During, or shortly after, the phlebotomy, a urine collection was made for measurements of renal function which were designated as obtained "during phlebotomy." In most of the patients additional

[1] This investigation was supported in part by a grant from the National Heart Institute of the National Institutes of Health, U. S. P. H. S.

[2] Present address: Queen's University, Kingston, Ontario, Canada.

TABLE I

Venous congestion

Patient Age / Sex / Body surface area (m^2)*	Clinical classification	Procedure	Duration (*min.*)	Periods	C_{PAH} *ml./min./1.73 m^2*	C_{IN} *ml./min./1.73 m^2*	FF %	UV *ml./min.*	$U_{Na}V$ *microEq./min.*	$U_{Cl}V$ *microEq./min.*	$U_{K}V$ *microEq./min.*	Hct. %	Arterial pressure§ *mm. Hg* S	D	M	P.R. *beats/min.*	Cardiac index *L./min./m^2*
A. S. / M / 1.95	A.S.H.D.† N.S.R. III C	Control	43	1–3	264	63	24	2.8	36	47	92	42	140	80	100	84	—
		Congestion	29	4–5	238	68	28	3.4	52	62	93	42	140	80	100	88	—
			15	6	259	69	27	3.8	75	80	105		130	75	90	88	—
			15	7	233	62	27	3.5	74	74	98	41	130	75	90	88	—
		Recovery	35	8–10	240	68	28	3.9	98	86	101	42	140	90	105	88	—
M. G. / F / 1.70	H.C.V.D. N.S.R. III C	Control	46	1–4	191	76	40	6.5	724	662	102	44	250	150	200	84	2.98
		Congestion	19	5–6	189	78	41	4.4	543	513	110		250	150	200	80	
			20	7–8	179	72	40	4.3	585	549	110		250	150	200	76	3.24 (21)‖
			21	9–10	208	81	40	4.4	648	598	118	45	250	150	200	76	3.13 (47)
		Recovery	28	11–13	207	80	39	3.2	491	460	102		250	150	200	76	—
L. G. / F / 1.52	R.H.D. A.S. N.S.R. III C	Control	46	1–3	180	82	46	0.4	26	58	24	36	95	65	75	104	2.00 1.99
		Congestion	20	4	198	84	42	0.5	50	97	33		95	65	75	100	
			28	5	193	80	41	0.5	46	98	37	35	95	65	75	96	2.11 (42)
J. D. / M / 1.65	H.C.V.D. N.S.R. IV E	Control	47	1–3	171	113	66	1.6	11	16	24	44	220	160	180	108	1.44
		Congestion	21	4	165	110	66	1.7	13	22	26		210	165	180	108	
			21	5	157	102	65	1.7	7	13	18		220	160	180	108	
			23	6	166	107	65	1.2	6	13	18	44	220	160	180	108	1.49 (50)
A. G. / M / 2.10	A.S.H.D. A.F. III D	Control	52	1–3	290	117	40	0.9	228	200	69	43	120	60	90	100	—
		Congestion	17	4	242	107	44	0.9	198	197	71		110	50	70	104	—
			26	5	192	82	42	0.6	114	119	52		100	40	60	100	—
			13	6	253	114	45	0.7	138	148	68	45	110	50	70	100	—
		Recovery	18	7	322	141	44	1.0	186	190	81		120	60	90		—
D. K. / M / 1.86	P.E. C.P. N.S.R. III C	Control	55	1–4	438	112	26	1.8	148	152	86	44	160	100	125	82	3.78
		Congestion	14	5	430	114	27	0.8	156	148	—		150	90	120	86	
			19	6	454	122	27	0.9	131	141	81	44	150	90	120	86	3.68 (28)
		Recovery	46	7–9	387	99	26	0.4	75	79	54		160	100	125	84	—
W. H. / M / 2.00	P.E. C.P.‡ N.S.R. IV E	Control	20	1–2	197	83	42	0.8	28	42	39	71	125	80	105	88	3.48
		Congestion	34	3–4	194	88	45	0.9	14	38	42		120	80	100	88	
			15	5	182	75	41	1.1	6	30	41		125	80	105	88	
			12	6	174	76	44	1.0	6	26	—	72	125	80	105	88	2.86 (59)
		Recovery	33	7–8	186	78	42	0.5	8	27	43		125	80	105	88	—
M. C. / F / 1.69	A.S.H.D. A.F. III C	Control	43	1–4	466	105	22	4.5	144	164	—	36	185	70	115	112	4.52 4.70
		Congestion	21	5–6	482	114	24	7.0	158	179	—	36	185	70	115	108	
			24	7–8	462	117	25	2.3	140	176	—		185	70	115	110	4.78 (31)
			26	9–10	415	109	26	1.5	105	136	—	35	185	70	115	108	4.57 (68)
		Recovery	20	11–12	401	109	27	1.3	82	112	—	36	185	70	115	108	4.82

* m^2 = Square meters of body surface area.

† R.H.D. = Rheumatic heart disease; A.S.H.D. = Arteriosclerotic heart disease; H.C.V.D. = Hypertensive cardiovascular disease; L.H.D. = Luetic heart disease; P.F. = Pulmonary fibrosis; P.E. = Pulmonary emphysema; C.P. = Cor pulmonale; A.F. = Auricular fibrillation; N.S.R. = Normal sinus rhythm; M.S. = Mitral stenosis; M.I. = Mitral insufficiency; A.I. = Aortic insufficiency; A.S. = Aortic stenosis; T.I. = Tricuspid insufficiency.

‡ During congestive failure.

§ S = Systolic; D = Diastolic; M = Mean.

‖ = Time in minutes at which cardiac output was measured from the beginning of the experimental procedure.

TABLE I—*Continued*

Patient Age Sex Body surface area (m^2)*	Clinical classification	Procedure	Duration (*min.*)	Periods	C_{PAH} *ml./min./1.73 m^2*	C_{IN} *ml./min./1.73 m^2*	FF %	UV *ml./min.*	$U_{Na}V$ *microEq./min.*	$U_{Cl}V$ *microEq./min.*	$U_{K}V$ *microEq./min.*	Hct. %	Arterial pressure‡ *mm. Hg* S	D	M	P.R. *beats/min.*	Cardiac index *L./min./m^2*
J. Di. 37 M 1.68	R.H.D. M.I. A.F. IV E	Control	26	1–2	237	114	48	9.1	31	29	28	38	110	65	80	42	1.64 1.77
		Congestion	14	3	234	120	51	8.5	37	31	26		120	70	85	48	
			17	4	162	78	48	2.4	20	20	15		118	65	82	48	1.86 (30)
			15	5	184	89	48	0.6	14	18	15	38	105	60	75	48	
		Recovery	63	6–8	248	116	47	0.8	17	25	24		110	65	80	48	—
G. B. 61 M 1.93	A.S.H.D. N.S.R. IV E	Control	32	1–3	—	91	—	7.1	165	96	77	38	160	60	95	85	—
		Congestion	32	4–5	—	77	—	1.3	82	43	61	37	170	65	100	85	
			14	6	—	86	—	1.2	68	30	53		160	50	90	88	—
			14	7	—	84	—	0.7	57	21	51	39	150	60	90	90	
		Recovery	90	8–10	—	79	—	0.8	95	35	60	39	160	60	95	84	—
R. O. 40 F 1.40	R.H.D. H.C.V.D. M.S. & M.I. A.F. IV E	Control	52	1–3	98	29	30	1.2	44	45	21	36	220	115	165	86	3.16
		Congestion	20	4	130	38	30	1.4	77	84	35		210	115	160	84	
			17	5	97	27	28	0.7	26	34	22		220	115	165	85	
			15	6	68	19	28	0.3	11	16	13	37	220	115	165	85	3.03 (43)
		Recovery	26	7–8	108	29	27	0.3	10	14	17		220	115	165	86	—
M. W. 62 F 1.63	R.H.D. M.S. A.F. IV D	Control	30	1–3	90	38	42	1.6	31	97	89	47	125	80	90	80	—
		Congestion	20	4–5	74	31	41	1.1	13	50	63	48	120	60	80	74	
			20	6–7	—	34	—	0.9	6	27	50		115	60	75	90	—
			20	8–9	—	29	—	0.4	3	12	35	49	120	60	80	90	
		Recovery	71	10–13	—	34	—	0.7	3	16	45	49	120	70	85	90	—
W. T. 39 M 1.89	R.H.D. A.S. & M.I. A.F. III D	Control	63	1–3	316	104	33	3.3	78	79	71	40	140	80	100	52	2.44 2.29
		Congestion	33	4–5	252	85	33	5.6	56	67	59		125	80	90	52	
			14	6	220	70	32	0.5	25	41	46		125	80	90	52	
			13	7	300	91	31	0.6	24	40	58	40	125	80	90	52	2.63 (53)
		Recovery	21	8–9	275	88	32	0.7	27	38	53		130	80	95	60	—
H. A. 50 M 1.74	L.H.D. A.I. N.S.R. III B	Control	75	1–4	153	38	25	3.4	77	56	37	37	250	105	160	84	3.34
		Congestion	12	5	170	45	26	3.3	92	83	49		210	85	125	80	
			10	6	92	23	25	0.8	34	29	22		205	85	120	80	
			19	7	50	12	23	0.2	15	—	12	36	195	95	125	76	3.11 (26)
		Recovery	46	8–9	219	49	23	1.6	15	16	39		215	100	135	80	—
R. H. 34 F 1.50	R.H.D. M.S. A.F. III C	Control	26	1–3	264	96	36	6.9	40	48	89	48	100	65	80	80	—
		Congestion	20	4–5	214	80	37	5.2	25	39	90	48	100	65	80	80	
			20	6–7	151	57	37	2.0	10	26	59		100	65	80	80	—
			20	8–9	99	36	37	0.4	4	13	32	50	100	65	80	80	
		Recovery	82	10–14	283	106	37	3.9	9	47	—	49	110	75	85	85	—

TABLE II

Phlebotomy

Patient Age Sex Body surface area (m^2)	Clinical classification	Procedure	Duration (*min.*)	Periods	C_{PAH} *ml./min./1.73 m^2*	C_{IN} *ml./min./1.73 m^2*	FF %	UV *ml./min.*	$U_{Na}V$ *microEq./min.*	$U_{Cl}V$ *microEq./min.*	$U_{K}V$ *microEq./min.*	Hct. %	Arterial pressure *mm. Hg* S	D	M	P.R. *beats/min.*	Cardiac index *L./min./m^2*
I. G. 63 M 1.76	A.S.H.D.* N.S.R. IV E	Control	60	1–3	139	—	—	0.3	9	11	34	46	160	100	120	82	—
		During phlebotomy (600 ml.)	(18)‡ 24	4	168	—	—	0.6	33	30	49	—	160	100	120	80	—
		After phlebotomy	52	5–7	115	—	—	0.6	48	50	54	42	160	100	120	80	—
W. D. 66 M 1.76	P.F. C.P.† N.S.R. III D	Control	104	1–4	176	78	45	1.0	9	31	55	50	130	70	100	80	3.70
		During phlebotomy (600 ml.)	(10) 28	5	240	99	41	1.3	16	41	60	46	130	70	100	80	2.77 (13)§
		After phlebotomy	66	6–9	175	83	48	0.9	29	53	46	47	130	70	100	80	—
M. W. 50 F 1.35	R.H.D. M.S. & M.I. & T.I. A.F. IV E	Control	214	1–3	—	—	—	0.3	2	2	1	50	140	90	110	80	1.16
		During phlebotomy (700 ml.)	(40) 40	4	—	—	—	0.2	2	—	1	45	120	70	80	80	—
		After phlebotomy	73	5–7	—	—	—	0.9	2	9	3	—	120	70	80	80	1.77 (54)
D. C. 48 M 1.91	H.C.V.D. N.S.R. III C	Control	85	1–6	245	74	30	8.0	236	251	49	44	210	90	140	86	4.80
		During phlebotomy (450 ml.)	(40) 40	7–9	256	77	30	4.1	270	313	69	43	210	85	140	90	—
		After phlebotomy	16	10	266	78	29	3.6	288	341	77	43	210	90	140	90	5.37 (50)
F. A. 61 F 1.85	A.S.H.D. N.S.R. III D	Control	17	1–5	156	60	38	7.4	38	45	43	55	120	70	85	86	2.21 2.00
		During phlebotomy (500 ml.)	(26) 21	6	147	59	40	1.4	43	65	41	53	110	70	85	84	—
		After phlebotomy	41	7–8	142	54	38	0.5	27	50	44	51	120	70	85	85	2.23 (34)
C. Di. 63 M 1.85	P.E. C.P. N.S.R. II B	Control	40	1–3	361	—	—	2.0	18	16	35	56	140	70	100	60	3.39
		During phlebotomy (600 ml.)	(10) 16	4	351	—	—	3.8	17	18	43	55	130	65	100	62	—
		After phlebotomy	30	5–8	306	—	—	0.9	8	12	33	—	130	65	100	62	2.98 (29)

* See Table I for explanation of abbreviations.
† During congestive failure.
‡ First figure is the duration of the phlebotomy.
§ Time in minutes at which cardiac output was measured from the beginning of the experimental procedure.

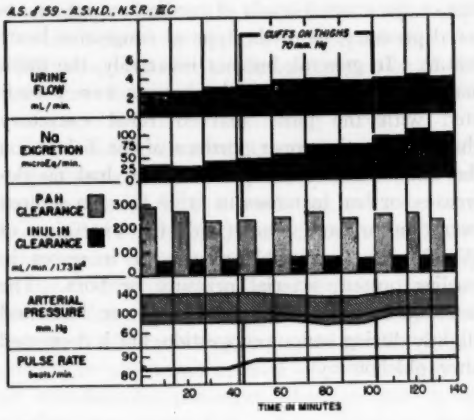

FIG. 1. CHART OF URINE FLOW, SODIUM EXCRETION, PAH CLEARANCE, INULIN CLEARANCE, ARTERIAL PRESSURE, AND PULSE RATE IN A PATIENT WITH ARTERIOSCLEROTIC HEART DISEASE AND CONGESTIVE FAILURE IN WHOM VENOUS CONGESTING CUFFS ON THE THIGHS WERE APPLIED FOR 60 MINUTES

Note the slight increase of urine flow, slight but progressive increase in the excretion of sodium, and the absence of significant change in the renal clearances or arterial pressure.

urine collections were made at 10 to 20-minute intervals after the release of the venous congesting cuffs or completion of venesection. In ten patients, measurements of cardiac output and central vascular pressures were repeated after 7 to 68 minutes of continuous venous congestion of the legs. In five patients, similar measurements were made from 3 to 20 minutes after the completion of venesection. Because of the difficulty of the determination, frequent measurements of cardiac output were impossible. They had to be "spotted" at various intervals when they were considered to be most desirable as judged from the general clinical condition of the patient (Tables I and II). Arterial pressures on the other hand were taken continuously.

All pressures were measured with electromanometers [3] and recorded by direct-writing oscillograph. Mean pressures were determined by electrical integration. Blood and urine concentrations of para-aminohippuric acid were measured by the method of Goldring and Chasis (3), and inulin was determined by the method of Schreiner (4) or Roe, Epstein, and Goldstein (5). Serum and urinary sodium and potassium were determined by the internal standard flame photometer (lithium standard). Serum and urinary chlorides were analyzed by the method of Wilson and Ball (6). The hematocrit was measured in Wintrobe tubes.

[3] Sanborn Company, Cambridge, Massachusetts.

RESULTS

Urine flow and sodium excretion

The responses to venous congestion of the legs are summarized in Table I, and those to phlebotomy in Table II. In both Tables the patients are listed in the approximate order of increasing degrees of antidiuretic response (see Discussion). Thus, at the top of Table I patients A. S., M. G., and L. G. had little or no fall in urine flow during venous congestion, and that two of them (A. S. and L. G.) had an increase in sodium excretion (Figure 1). Reading downward in Table I more definite antidiuretic responses are seen (Figure 2) and at the bottom are found the strong responses, usually seen in normal subjects, of patients W. T., H. A., and R. H., all of whom had definite decreases in both urine flow and sodium excretion during venous congestion of the limbs.

Table II is arranged similarly and demonstrates

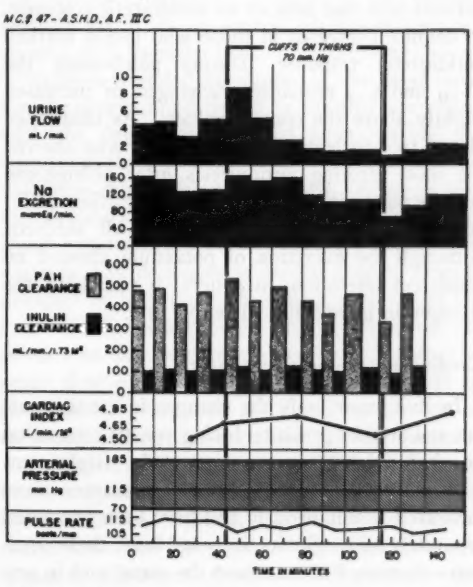

FIG. 2. CHART OF AN EXPERIMENT IN A PATIENT WITH ARTERIOSCLEROTIC HEART DISEASE AND CONGESTIVE FAILURE CONDUCTED SIMILARLY TO THAT SHOWN IN FIGURE 1 EXCEPT THAT THE ADDITIONAL MEASUREMENT OF CARDIAC OUTPUT WAS MADE

Note the definite and progressive reductions in urine flow and the excretion of sodium, slight decreases in PAH clearance, and the insignificant changes in cardiac output and arterial pressure.

that patients who had sizable venesections showed somewhat less marked reductions in urine flow than those who had definite antidiuretic responses to venous congestion of the extremities. None of the patients in this group had a reduction in the excretion of sodium (or chloride) *during* phlebotomy; in fact, patients I. G., W. D., D. C. actually appeared to have increases in the rate of excretion of sodium. However, *after* the completion of the phlebotomy (when, of course, the blood volume was presumed to be most reduced), the excretion of sodium decreased in two patients (F. A., C. Di.) while the renal plasma flow decreased slightly, along with the glomerular filtration rate (in patient F. A.).

Renal hemodynamic responses

Changes in renal plasma flow (C_{PAH}) and glomerular filtration rate (C_{IN}) during (and after) venous congestion ranged from none or slight in patients who had little or no antidiuretic response, to definite decreases in those who had a marked antidiuretic response. *During* phlebotomy the C_{PAH} and C_{IN} remained unchanged or increased slightly above the control values. As mentioned above, two patients (F. A., C. Di.) who showed the most striking antidiuresis *after* phlebotomy had decreases in C_{PAH}. Filtration fraction (FF) remained essentially unchanged in all subjects. Although the excretion of potassium showed no consistent alteration, in general it paralleled the changes in glomerular filtration rate.

Cardiohemodynamic responses

In this paper, only the changes in cardiac output and arterial pressure during venous congestion or after phlebotomy are presented. Right ventricular and pulmonary arterial pressures were measured simultaneously and have been presented separately (2). Suffice it to say here, these pressures decreased or remained the same, and in any case did not show any consistent correlation with changes in cardiac output or renal excretion.

As seen in the lower portions of Tables I and II in those patients showing definite decreases in urine flow and excretion of sodium during venous congestion or after phlebotomy, either a reduction or no change was observed in cardiac output, arterial pressure, or both. This was true regardless of the control levels of cardiac output or arterial pressure, or of the type of congestive heart failure. In general, but not invariably, the more marked cardiohemodynamic changes were associated with the more marked renal excretory changes. In the upper portions of the Tables, on the other hand, those patients who had no decreases, or had increases in urine flow, in sodium excretion or both, had (with the exception of W. D.) no appreciable change or increases in cardiac output, arterial pressure or both. The hematocrit either did not change or increased slightly during venous congestion, but it decreased after phlebotomy.

DISCUSSION

Previous studies have indicated that the decreases in urine flow and sodium excretion occurring during venous congestion of the limbs of normotensive and compensated hypertensive subjects are consequences of a reduction in the effectively circulating blood volume (1). These responses do not appear if the reduction is offset by a large transfusion given simultaneously with the congestion of the limbs (7). Therefore, the responses appeared to be related to the homeostatic mechanisms tending to counteract the effects of a suboptimal effectively circulating blood volume. This view was supported by the observation that these responses were particularly pronounced and prolonged if a clinical state of collapse of the circulation occurred, with hypotension, anxiety, pallor, sweating, nausea, or faintness.

It was of considerable interest to examine what and where the stimuli may be that call forth these responses which occur quite regularly in normotensive and compensated hypertensive subjects. Cardiohemodynamic studies in such subjects indicate that congesting the limbs usually decreases the cardiac output without consistently causing measurable changes in systemic or pulmonary arterial pressure or right ventricular end-diastolic pressure (2). Any decrease in cardiac output during venous congestion therefore appeared usually to indicate a reduction in effectively circulating blood volume, which if it were not counteracted by vasomotor (and other homeostatic) adjustments would result in definite arterial hypotension, and indeed does so when the

vasomotor reflexes are blocked by drugs (8). Therefore the hypothesis was accepted that the antidiuretic responses to venous congestion of the limbs are homeostatic reactions to suboptimal amounts of effectively circulating blood.

By extending the study to cardiac patients with congestive failure it was possible further to test this hypothesis. Thus it was found, as others have shown (9, 10), that certain patients in congestive failure have increases in cardiac output during venous congestion of the limbs, whereas other patients in failure, like normal subjects, may have decreases. Since both groups had similar or no changes in pulmonary arterial and right ventricular end-diastolic pressures, the question arose whether any connection existed between their cardiohemodynamic responses and their renal excretory responses. To make this comparison, it was necessary first to grade the renal excretory responses. This was done (in Tables I and II) on the basis of the following general considerations. The antidiuretic response was considered to be definite provided the control rate of urine flow was over 2 ml. per minute (and preferably over 3 ml. per min.) and if: 1. A decrease in urine flow occurred to or below 25 per cent of the control rate and to or below 2 ml. per minute; 2. A decrease in sodium excretion occurred to or below 75 per cent of the control rate.

A definite decrease in sodium excretion was considered a stronger response physiologically than a definite decrease in urine flow because reduced sodium excretion was never seen in normal subjects in the absence of a definite reduction in urine flow, whereas the reverse was seen not infrequently. Therefore, if only a moderate decrease of urine flow occurred (to 50 per cent of the control) the antidiuretic response was regarded as slight whereas if urine flow decreased to 10 per cent and sodium excretion to 50 per cent the response was regarded as strong. Only rarely was a slight response found to occur in a normal subject, and an *increase* in urine flow or sodium excretion or both was never seen in a normal subject or in a compensated hypertensive patient and would have been regarded as most unusual in this group.

One other factor in addition to the nature and degree of these responses was considered of importance in grading them. This was the *persistence* of the changes observed. Thus after collapse of the circulation, the responses not only were severe and included reductions in urine flow, sodium excretion, renal plasma flow and glomerular filtration rate but they persisted for some time after the stimulus and recovered in an order the reverse of that just listed, sometimes only after an hour.

Obviously from these considerations all gradations of renal excretory responses would be conceivably possible, from a definite antidiuresis to an actual diuresis of water and sodium. Indeed every gradation of response was seen in patients with congestive failure. Thus in Table I, patient A. S. (at the top) had an increase in the rates both of urine flow and of sodium excretion, while patients M. G. and L. G. had no change in either as defined above. Patient M. C. (in the middle of the table) had a definite reduction in urine flow and a moderate decrease in sodium excretion during venous congestion. Finally at the bottom, patients W. T., H. A., and R. H. had definite decreases in both urine flow and sodium excretion.

As already stated the degree of the renal functional changes appeared to be roughly correlated with the degree of the cardiohemodynamic responses to these stimuli. Thus, patients who had a definite antidiuretic response (in the lower part of the Tables), like normal subjects and compensated hypertensive patients, usually, but not invariably, had decreases in cardiac output, arterial pressure or both during venous congestion of the limbs or after phlebotomy. In sharp contrast, patients who had no decrease or had an increase in urine flow or sodium excretion (upper part of the Tables) either had no significant change or an increase in cardiac output and arterial pressure.

There were no consistent qualitative differences in the responses of the patients whose effectively circulating blood volumes were reduced by venous congestion of the limbs and of those who had venesections. However, there were quantitative differences in that venous congestion more frequently caused measurable decreases in cardiac output and definite decreases in the renal excretion of water and salt. Thus, at least half of the patients in failure who had venous congestion of the limbs had definite antidiuretic responses while no more than a third of those who had phlebotomy had such responses.

These quantitative differences in the effects of venous congestion and of phlebotomy may be related to the quantitative differences between the amount of blood removed from active circulation in each case. It is estimated that venous congestion, as employed in these experiments, pools somewhat more than 1,000 ml. of blood in the limbs whereas the largest phlebotomy performed was 700 ml. It seems logical, and it has been repeatedly observed clinically, that in patients with severe congestive failure, a moderate reduction in effective circulating blood volume may result in subjective relief and evidences of circulatory improvement, but that too great a reduction in volume may cause rapid deterioration of the circulation even to the point of circulatory collapse. Thus there presumably is an optimal effectively circulating blood volume for each individual patient, deviations from which, either above or below the optimum, would bring unfavorable consequences.

This assumption is compatible with the observations made in the present study. It was repeatedly observed that when arterial pressure fell or became unusually variable, when the arterial wave fronts assumed a peaked "collapse" form with a low or absent dicrotic notch and a flat diastolic run-off, or even more important, when the patient became anxious, pale, sweaty, nauseous, or faint definite antidiuresis and reduction in sodium excretion would follow. Occasionally collapse occurred (as in patient J. Di.) even though cardiac output was found not to be decreased when it was measured. For these reasons, reductions in both arterial pressure and cardiac output were regarded as important, but not the only important, objective signs of imminent collapse. Pulse rate acceleration in these patients with congestive failure was a less reliable sign of inadequate circulating blood volume than in normotensive or compensated hypertensive subjects. Presumably this was because of digitalization of the patients, and also because of the associated arrhythmias present in many of them.

It is clear from the above considerations that we have come to regard a clinical state of imminent circulatory collapse as the important stimulus to the renal excretory changes of reduced urine flow, reduced sodium excretion, and (at least initially) decreased renal plasma flow, and glomerular filtration rate. In general this state may be signaled by reductions in cardiac output, arterial pressure or both. It certainly is not invariably accompanied by a reduction in right ventricular or pulmonary arterial pressure. Circulatory collapse may be said to be imminent, or possible, whenever there is a suboptimal, hypovolemic, and hypokinetic state of the circulation. If this is a reasonable concept, it may account for the fact that the only group of patients in congestive failure who showed a poor correlation between reductions in cardiac output and reductions in water and salt excretion during the hypovolemic procedure employed were those with cor pulmonale and so-called "high output" failure. Thus patient W. H. during venous congestion and patient W. D. after phlebotomy had sizable reductions in cardiac output, arterial pressure or both, but only a relatively small decrease in urine flow or sodium excretion. It seems possible that these patients actually had a more "normal" circulation during the hypovolemic procedure than before. One such patient, C. Di., however, did show the usual antidiuretic response when the cardiac output and arterial pressure were reduced.

The explanation just offered, namely that a suboptimal, hypovolemic, hypokinetic state of the circulation is the effective stimulus to the antidiuretic response, is also compatible with the observation that closing a large, long-established arteriovenous shunt usually causes an increase in sodium and water excretion, whereas opening a new AV shunt in dogs generally causes a decrease in water and salt excretion (11). In these latter experiments the changes in right cardiac and pulmonary arterial pressures are just the opposite to those found in hypovolemic experiments, but the systemic arterial pressures, and indeed the danger of circulatory collapse, and the renal excretory responses are similar. Although it is true that the cardiac output increases in the shunt-opening experiments, and decreases in the blood-volume-reducing experiments, *relative to demand* it may be presumed to be suboptimal in both. Therefore, no single cardiohemodynamic measurement in itself can be taken as uniformly indicative of a suboptimal circulation. Only when the supply of effectively circulating blood *relative to demand* is adequate can the circulation be said to be optimal. A suboptimal state of the circulation undoubtedly

sets in motion a battery of compensating homeostatic mechanisms. One of these appears to be a relative decrease in water and salt excretion.

SUMMARY

1. In response to venous congestion of the legs or to a phlebotomy of 450 to 700 ml. of blood, patients with congestive heart failure may or may not have the decreases in water and salt excretion which normally occur in individuals without heart failure.

2. Simultaneous cardiohemodynamic measurements suggest that decreases in sodium and water excretion are more apt to occur after venous congestion or bleeding when there is a decrease in cardiac output or peripheral arterial pressure or clinical signs of imminent circulatory collapse. Increases in sodium and water excretion are more apt to occur after the same procedures if there is an increase in cardiac output or peripheral arterial pressure.

3. No apparent relationship was noted between the renal responses and changes in pulmonary arterial and right ventricular pressures.

4. The observed decreases in renal function after venous congestion of the legs or phlebotomy may be regarded as homeostatic responses to a suboptimal effectively circulating blood volume.

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PLASMA AND URINARY STEROIDS AFTER HYDROCORTISONE INFUSION[1]

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The experiments presented in this report were designed to determine the rate of disappearance of intravenously administered hydrocortisone in patients with Addison's disease before routine steroid therapy had been initiated, during therapy and after therapy had been withdrawn. The results of a similar study have been reported recently by Hellman, Bradlow, Adesman, Fukushima, Kulp, and Gallagher (1). These workers measured changes in radioactivity of the blood following the infusion of C^{14}-labelled hydrocortisone. We have analyzed plasma for hydrocortisone by a method which combines resolution on a silica gel column with quantitation by fluorescence. We have also determined the rate of excretion of those free and conjugated steroids which have a 17,21-dihydroxy-20-ketone group.[2]

METHODS

One hundred mg. of hydrocortisone[3] (free alcohol) in 500 ml. of 5 per cent glucose were infused into an antecubital vein during a period of 20 to 40 minutes. Plasma hydrocortisone was analyzed by the fluorescent method of Sweat (3, 4). Urine samples were collected via indwelling catheters and analyzed for free and conjugated 17,21-dihydroxy-20-ketones by the method of Glenn and Nelson (5). Plasma and urine samples were obtained for steroid analyses at the times designated in Tables I, II, and III. The fluorescent method is relatively specific for hydrocortisone (4) and, in the present study, where high levels of hydrocortisone were induced, is undoubtedly a reliable index of the concentration of this steroid in peripheral blood. The method for urinary steroids measured the total quantity of those corticosteroids with hydroxy at positions 17 and 21, and ketone at 20; the phenylhydrazine reagent reacts with these substances to give an absorption maximum at 400 mμ. An important feature of the method is the use of a Florisil column to eliminate the noncorticosteroid phenylhydrazine-reactive materials which are present in a chloroform extract of urine.

Three infusion studies were conducted on each of two patients with Addison's disease and one on a patient with presumably normal adenohypophyseal and adrenocortical function. The *first infusion* of hydrocortisone was given before replacement therapy was instituted. The *second infusion* was given approximately one month later during which time the patient received oral hydrocortisone therapy, 5 mg. every six hours. Four hours elapsed between the last oral dose of hydrocortisone and the infusion. The *third infusion* was given 48 hours after the second infusion; the patients received no steroid between the second and the third infusion tests.

Patient V. A 42-year-old Negro was admitted with pulmonary tuberculosis. He exhibited signs and symptoms which suggested adrenocortical insufficiency. Intravenous ACTH failed to induce an eosinopenia or to increase peripheral blood corticosteroids. The patient became acutely ill (pulse 160 to 180; blood pressure 70/52; disoriented; febrile) and he was given the *first infusion* of 100 mg. of hydrocortisone. He had not received steroid prior to this infusion. The patient's condition remained serious one hour after completion of hydrocortisone administration and he was given a supplementary dose of steroid (100 mg. hydrocortisone in 500 ml. of 5 per cent glucose).

Patient J. A 46-year-old male Negro exhibited the characteristic signs and symptoms of Addison's disease. Intravenous ACTH failed to induce an eosinopenia or to increase peripheral blood corticosteroids. On April 18, patient J received 100 mg. of cortisone intramuscularly. The *first infusion* was given on April 29th.

Patient R had apparently normal adrenal and pituitary function. The patient had a congenital cardiac anomaly and was asymptomatic throughout the experimental period. The patient received no steroid prior to infusion of hydrocortisone.

[1] This investigation was supported by a grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

[2] The results of this study were presented at the Fall Meeting of the American Physiological Society, Madison, Wisconsin, September, 1954 (2).

[3] Generous gifts of Dr. H. F. Hailman of the Upjohn Company and of Dr. E. Alpert of Merck and Company.

TABLE I

Plasma and urine steroids before and after hydrocortisone (F) infusion in a patient with Addison's disease (patient V)

Time *min.*	Plasma F *μg./100 ml.*	Urine					
		Volume		Free steroids		Conjugated steroids	
		ml.	*ml./min.*	*μg.*	*μg./min.*	*μg.*	*μg./min.*
First infusion							
6-hr. pre-F urine collection		615	1.7	0	0	0	0
5 min. pre-F	5	—	—	—	—	—	—
0 100 mg. F infused	—						
30	138						
35	132	300	4.0	1,300	17	4,020	54
45	124						
60	—						
75 100 mg. F infused	—						
105	210						
110	214	480	4.0	1,200	10	2,200	18
120	196						
165	—						
195	148						
240	—	310	2.6	3,100	26	7,600	64
315							
Second infusion							
24-hr. pre-F urine collection		2,510	1.7	0	0	0	0
30 min. pre-F	13	—	—	—	—	—	—
0 100 mg. F infused	110	40	2.7	154	10	585	39
15		50	3.3	520	35	920	61
30	156						
60	131	180	6.0	1,194	40	2,940	98
90	125	120	4.0	420	14	2,520	84
150	60	290	4.8	738	12	3,210	54
270	41	430	3.6	864	7.2	6,804	57
510	24	370	1.5	288	1.2	2,328	9.7
990	5	540	1.1	0	0	0	0
1,490	3	466	0.9	0	0	0	0

TABLE I—*Continued*

Time min.		Plasma F μg./100 ml.	Urine Volume ml.	Urine Volume ml./min.	Urine Free steroids μg.	Urine Free steroids μg./min.	Urine Conjugated steroids μg.	Urine Conjugated steroids μg./min.
Third infusion								
24-hr. pre-F urine collection			2,476	1.9	0	0	0	0
5 min. pre-F		5	—	—	—	—	—	—
0	100 mg. F infused							
15		76	20	1.3	120	8.0	624	42
30		106	30	2.0	592	39	532	35
60		73	80	2.7	960	32	2,703	90
103		54	80	1.9	375	8.7	1,620	38
150		35	200	4.3	1,289	27	4,296	91
270		40	500	4.6	864	7.2	5,136	43
510		25	600	2.5	264	1.1	5,904	25
990		11	430	0.9	0	0	0	0
1,410		7	375	0.9	0	0	0	0
2,950		7	2,365	1.6	0	0	0	0

TABLE II

Plasma and urine steroids before and after hydrocortisone (F) infusion in a patient with Addison's disease (patient J)

Time min.		Plasma F μg./100 ml.	Urine Volume ml.	Urine Volume ml./min.	Urine Free steroids μg.	Urine Free steroids μg./min.	Urine Conjugated steroids μg.	Urine Conjugated steroids μg./min.
First infusion								
4-hr. pre-F urine collection			510	2.1	0	0	0	0
5 min. pre-F		5	—	—	—	—	—	—
0								
20	100 mg. F infused	119	70	1.8	600	15	800	20
40		—						
52		145						
64		145	60	1.3	700	15	2,100	44
88		144						
290		40	480	2.4	800	4.0	6,800	34
1,460		4	1,820	1.6	0	0	0	0
2,900		—	4,060	2.8	0	0	0	0

TABLE II—*Continued*

Time *min.*	Plasma F *μg./100 ml.*	Urine					
		Volume		Free steroids		Conjugated steroids	
		ml.	*ml./min.*	*μg.*	*μg./min.*	*μg.*	*μg./min.*
Second infusion							
24-hr. pre-F urine collection		1,608	1.1	158	0.1	3,124	2.2
20 min. pre-F	17	—	—	—	—	—	—
0							
15 100 mg. F infused	66	90	3.0	960	32	1,140	38
30	132						
60	89	30	1.0	2,295	77	1,650	55
90	58	40	1.3	942	31	2,580	86
150	61	150	2.5	1,596	27	4,680	78
270	28	175	1.5	792	6.6	6,980	57
457	15	150	0.8	384	2.1	3,120	17
937	3	238	0.5	768	1.6	1,014	2.1
1,417	2	530	1.1	0	0	0	0
Third infusion							
24-hr. pre-F urine collection		1,723	1.2	0	0	0	0
5 min. pre-F	<2	—	—	—	—	—	—
0							
11 100 mg. F infused	132	85	3.9	556	25	396	18
22	165						
52	120	50	1.7	660	22	1,230	41
82	101	50	1.7	708	24	3,510	117
142	53	70	1.2	780	13	4,710	78
262	27	800	6.7	1,016	8.4	9,840	82
502	22	800	3.3	264	1.1	7,440	31
982	13	282	0.6	34	0.07	720	1.5
1,440	<2	330	0.7	0	0	0	0
2,880	<2	1,535	1.1	0	0	0	0

TABLE III

Plasma and urine steroids before and after hydrocortisone (F) in a patient with normal adrenal function (patient R)

Time min.		Plasma F μg./100 ml.	Urine					
			Volume		Free steroids		Conjugated steroids	
			ml.	ml./min.	μg.	μg./min.	μg.	μg./min.
24-hr. pre-F urine collection			1,290	0.9	0	0	4,032	2.8
5 min. pre-F		13	—	—	—	—	—	—
0	100 mg.							
15	F	94	55	3.6	406	27	807	54
30		102	24	1.6	696	46	766	51
60		54	112	3.7	552	18	2,676	89
90		40	140	4.7	420	14	1,440	48
150		60	315	5.3	720	12	3,060	51
270		45	155	1.3	480	4.0	5,040	42
510		13	190	0.8	336	1.4	5,520	23
990		8	320	0.7	48	0.1	4,512	9.2
1,470		15	360	0.8	48	0.1	1,392	2.9
2,910		12	1,228	0.9	0	0	187	0.1

RESULTS AND DISCUSSION

Pre-infusion levels of plasma hydrocortisone

In the patients with Addison's disease the highest pre-infusion level of plasma hydrocortisone was obtained prior to the second infusion and was of the same order of magnitude as that obtained in the subject with normal adrenals (Tables I, II, and III).

Values of 5 μg. per 100 ml. were obtained in the patients with Addison's disease at a time when they had not been treated with hormone therapy. These results must be interpreted with caution. Although the problem of steroid concentration in Addison's disease as compared to normal subjects is not the subject of this study a short comment is in order. On the one hand, a substance in plasma may interfere and give a constant background reading in the analyses of hydrocortisone. On the other hand, patients with Addison's disease may vary in regard to the degree of adrenocortical insufficiency. Small masses of adrenocortical tissue may secrete steroid in sufficient quantity to maintain fixed minimal levels of hydrocortisone. This tissue may be presumed to be secreting at a maximum rate at all times. In response to a stressful stimulus no increase in rate of production of steroid will occur. Eik-Nes, Sandberg, Migeon, Tyler, and Samuels (6) have observed patients with Addison's disease who exhibited normal levels of 17-hydroxycorticoids. These patients did not respond to ACTH with an elevation of blood steroid. The two patients observed in this study also did not respond to ACTH with an elevation of blood steroids (7). The problem has important implications from the standpoint of micromethods for analysis of steroids and from the standpoint of the pathological physiology of Addison's disease. No definite conclusion may be made at this time. Additional studies must be conducted to test the absolute specificity of the micromethods at low concentrations of blood steroids.

Infusion levels of plasma hydrocortisone

As expected, the concentration of hydrocortisone in the plasma increased rapidly during infusion of 100 mg. of this steroid over a period of 30 minutes (Figures 1–7). The average concentration was 135 μg. per 100 ml. at the end of the infusion in seven tests. (Included in the average are two samples, one taken at 5, the other at 12 minutes after the end of the infusion.) In the patients with Addison's disease the levels of hydrocortisone at the end of the first infusions (138, 145 μg. per 100 ml.) do not appear to be significantly different from those obtained at the end of the second (156, 132 μg. per 100 ml.) or at the end of the third infusion (106, 165 μg. per 100 ml.). The concentration of hydrocortisone at the end of infusion in the normal subject, 102 μg. per 100 ml., was the lowest value obtained. The tentative conclusion appears warranted that a deficiency of cortical steroids is not associated with an accelerated rate of disappearance of hydrocortisone from the plasma when a large dose of the steroid is infused over 30 minutes.

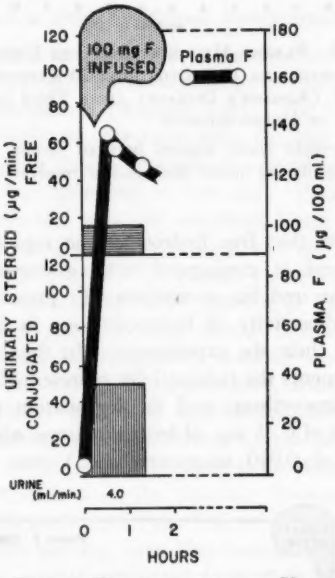

FIG. 1. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT V (ADDISON'S DISEASE) AFTER *First Infusion* OF 100 MG. OF HYDROCORTISONE

Patient had received no steroid therapy prior to first infusion.

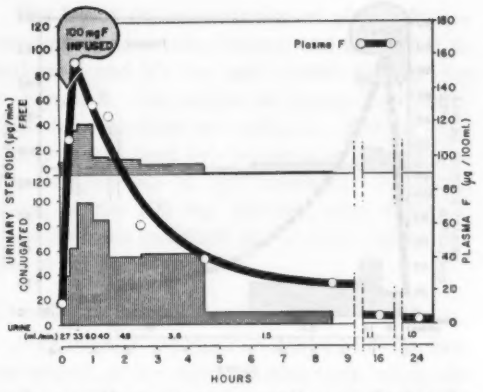

FIG. 2. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT V (ADDISON'S DISEASE) AFTER *Second Infusion* OF 100 MG. OF HYDROCORTISONE

Patient was on hydrocortisone therapy (5 mg. every six hours) for one month between first and second infusions.

The rapidity of exit of infused hydrocortisone from the vascular compartment is dramatically illustrated by a simple calculation. The average concentration of hydrocortisone at the end of infusion was 135 μg. per 100 ml. or 1.35 mg. per liter. The total quantity in the plasma at the end

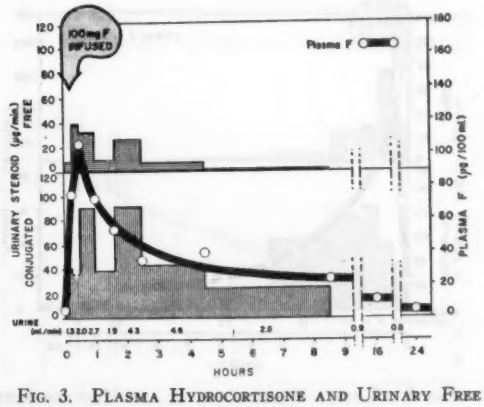

FIG. 3. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT V (ADDISON'S DISEASE) AFTER *Third Infusion* OF 100 MG. OF HYDROCORTISONE

Forty-eight hours elapsed between second and third infusions during which time patient received no steroid therapy.

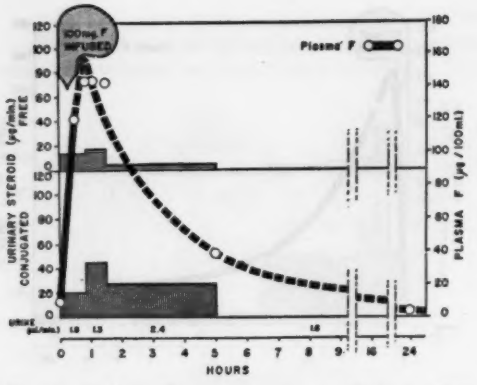

FIG. 4. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT J (ADDISON'S DISEASE) AFTER *First Infusion* OF 100 MG. OF HYDROCORTISONE

Patient had received only one dose of steroid prior to first infusion (100 mg. of cortisone were given intramuscularly 11 days before the first infusion).

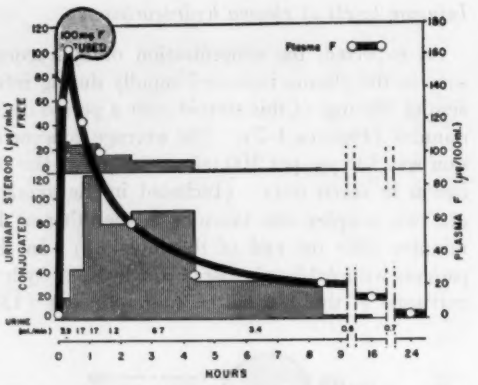

FIG. 6. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT J (ADDISON'S DISEASE) AFTER *Third Infusion* OF 100 MG. OF HYDROCORTISONE

Forty-eight hours elapsed between second and third infusions, during which time patient received no steroid therapy.

of the infusion equals 1.35 mg. per liter times 3 liters (plasma volume), or 4 mg. Hence, of the 100 mg. infused, only 4 mg. remained in the total volume at the end of the 30-minute infusion. Less than 2 mg. of free steroid was excreted during the infusion. (See free steroid excretion data in Tables I, II, and III.) The fact that steroid glucuronides were excreted during the infusion suggests that free hydrocortisone rapidly enters cells and is conjugated with glucuronic acid. Hellman and his co-workers (1) presented data for radioactivity of hydrocortisone in blood for one of their six experiments. In the other five experiments the radioactivity represented the sum of hydrocortisone and its degradation products. A dose of 0.25 mg. of hydrocortisone with an activity of 0.940 microcurie (μc.) was injected.

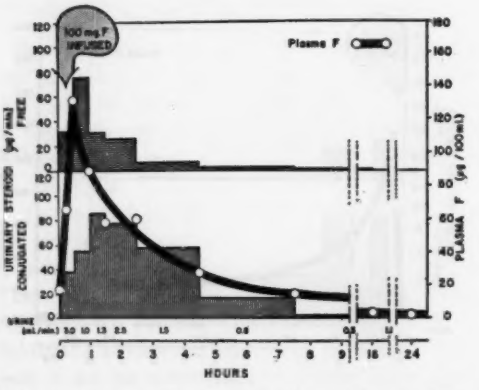

FIG. 5. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT J (ADDISON'S DISEASE) AFTER *Second Infusion* OF 100 MG. OF HYDROCORTISONE

Patient was on hydrocortisone therapy (5 mg. every six hours) for one month between first and second infusions.

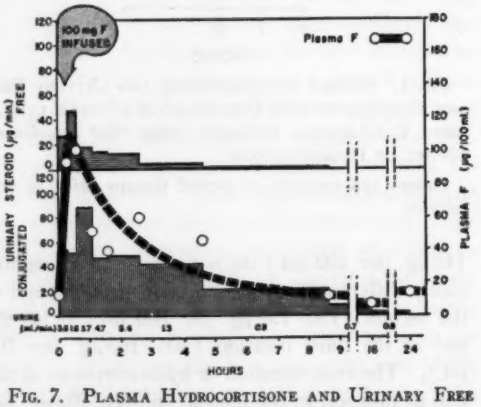

FIG. 7. PLASMA HYDROCORTISONE AND URINARY FREE AND CONJUGATED 17,21-DIHYDROXY-20-KETONES IN PATIENT R (NORMAL PITUITARY-ADRENOCORTICAL FUNCTION) AFTER INFUSION OF 100 MG. OF HYDROCORTISONE

Patient had not received steroid therapy prior to infusion.

Fifteen minutes after the end of the infusion the activity of hydrocortisone plus degradation products in blood was 0.021 μc. per liter. For a blood volume of 6 liters total activity in the vascular compartment equals .126 μc. A maximum of 15 per cent of this radioactivity was present as unaltered hydrocortisone. Therefore, approximately 2 per cent of the injected hydrocortisone was present after 15 minutes, a figure which agrees with our values obtained in 7 experiments. Our observations are also in agreement with those of Brown, Willardson, Samuels, and Tyler (8).

Post-infusion levels of plasma hydrocortisone

The post-infusion period is characterized by a rapid reduction in the concentration of hydrocortisone in the plasma (Figures 1–7). The half-life of hydrocortisone, the time after the end of infusion for the plasma concentration to decrease to one-half, was estimated in four tests: Patient V, second infusion, two and one-third hours (Figure 2); patient V, third infusion, one and three-fourth hours (Figure 3); patient J, second infusion, two hours (Figure 5); patient J, third infusion, one and one-third hours (Figure 6). The estimates are remarkably uniform when one considers the complexity of processes, urinary excretion, biliary excretion, degradation, diffusion, selective concentration in certain tissues and conjugation, which must be involved in the disappearance of steroids from the plasma. Brown, Willardson, Samuels, and Tyler infused 1 mg. of hydrocortisone per Kg. over 30 minutes. The half-life of the steroid estimated from their observations is in excellent agreement with the results of this study.

Unfortunately, adequate numbers of samples were not obtained in the first infusion tests on the patients with Addison's disease for accurate estimates of half-life. Patient V was given a supplementary infusion at the first test which complicated the study (Figure 1). In patient J, the half-life in the first infusion test as estimated from the limited data available does not appear to be less than that of the patients with Addison's disease in the second or third tests (Figure 4). The post-infusion values for plasma hydrocortisone in the patient with normal adrenals (patient R) do not follow a consistent enough pattern to warrant the estimation of half-life (Figure 7).

The log of the concentration of plasma hydrocortisone in four tests (second and third tests on patients J and V) has been plotted against time in Figure 8. No simple rectilinear relationship exists between these two variables. The curve indicates that at least two processes are involved in the disappearance of hydrocortisone. As stated above, of the 100 mg. injected, only 4 mg. of hydrocortisone remained in the total plasma volume at the end of infusion.

Urinary excretion of steroids

Free steroids. In the 24 hours prior to the first, the second, or the third infusion tests, no significant quantity of free 17,21-dihydroxy-20-ketones was excreted by any subject except patient J, who excreted 158 μg. during the 24 hours prior to the second test (Tables I, II, and III). Patient R with normal adrenal function did not excrete a detectable quantity of free steroid in the 24 hours prior to the infusion. These observations indicate that exceedingly small quantities of hydrocortisone and related steroids are excreted in the free form in normal subjects or in patients with Addison's disease given maintenance doses of hydrocortisone.

The peak rate of excretion of free 17,21-dihydroxy-20-ketones was attained during the infusion of hydrocortisone or in the 30-minute collection period which immediately followed (Tables I, II, and III, and Figures 1–7). The subsequent values for rate of excretion of free steroids paral-

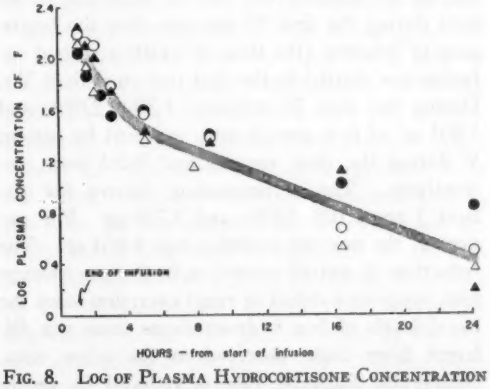

FIG. 8. LOG OF PLASMA HYDROCORTISONE CONCENTRATION PLOTTED AGAINST TIME FOR FOUR INFUSION TESTS

○—second infusion in patient V; ●—third infusion in patient V; △—second infusion in patient J; ▲—third infusion in patient J.

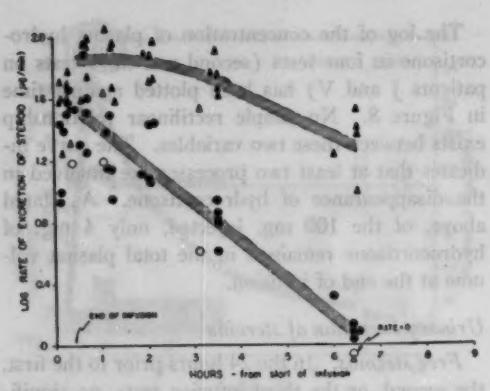

FIG. 9. LOG OF THE RATE OF URINARY EXCRETION OF FREE AND OF CONJUGATED 17,21-DIHYDROXY-20-KETONES PLOTTED AGAINST TIME

●—free and ▲—conjugated steroids for all infusions except the first infusion in patient J, in which designations are ○ and △.

leled the plasma levels of hydrocortisone except for the third infusion test on patient V for the 60 to 103-minute period following infusion.

Approximately 4 per cent of the administered steroid can be accounted for as free 17,21-dihydroxy-20-ketones excreted in the 24 hours following the infusion. Patient J excreted a smaller quantity of free steroid during the first test as compared to the amounts excreted during the second and third tests.

The first test on patient V was complicated by a second infusion of steroid. However, it is of interest to compare the rate of excretion in all tests during the first 75 minutes after the beginning of infusion (the time at which a second infusion was started in the first test on patient V). During the first 75 minutes, 1,300, 2,078, and 1,803 μg. of free steroid were excreted by patient V during the first, second, and third tests, respectively. The corresponding figures for patient J are 1,105, 3,726, and 1,768 μg. For patient R the amount excreted was 1,864 μg. The reduction of steroid excretion in the pre-therapy tests suggests a defect in renal excretion since the blood levels of free hydrocortisone were not different from those observed in the other tests. Glomerular filtration rate is reduced in severe adrenocortical insufficiency (9) and may account for the observed reduction in the rate of excretion of free steroids. In Figure 9, log of the rate of excretion of free steroids in micrograms per minute has been plotted against time. The estimated time for the process to be reduced to one-half its peak rate is one and one-half hours, a value which corresponds quite closely with the half-life of hydrocortisone in the plasma.

Conjugated steroids. No significant quantity of conjugated steroid was excreted by the two patients with Addison's disease in the pre-infusion periods except in the case of patient J who excreted 3,124 μg. in the 24 hours prior to the second test (Tables I and II). The subject with normal adrenals excreted 4,032 μg. (Table III). No explanation can be offered as to why patient V did not excrete conjugated steroids at a time when he was receiving 5 mg. of hydrocortisone by mouth, six times per day.

That the process of conjugation occurs at a remarkably rapid rate is indicated by the fact that conjugated steroids appeared in the urine in relatively large quantities during the first 15 minutes of the infusion (see Tables and Figures). However, the peak rate of excretion of the conjugated steroids usually occurred in the period immediately following the establishment of the peak rate of excretion of the free steroids.

The conjugates excreted during the 24 hours following infusion accounted for approximately 20 per cent of the injected steroid. Patient J excreted only 9.7 per cent of the administered steroid as conjugates in the first test as compared to 21.2 and 27.8 per cent in the second and third tests. The patient with normal adrenals excreted 25.4 per cent of the infused hydrocortisone.

During the first 75 minutes after the start of the infusion, patient V excreted 4,026, 5,705, and 4,429 μg. in the first, second, and third tests, respectively. The corresponding figures for patient J were 2,340, 4,080, and 4,317 μg. Patient R excreted 4,969 μg. As in the case of the free steroid, there appears to be a reduction in renal excretion of conjugated steroids in subjects with Addison's disease given intravenous hydrocortisone at a time when they have had no previous steroid therapy. The reduced rate of excretion could be due to a renal impairment or to a defect in the rate of conjugation of hydrocortisone. According to Hellman and his associates (1), renal excretion of total steroids as measured by radioactivity was normal in one patient with Addison's disease who

had received no therapy for 48 hours prior to infusion of hydrocortisone-4-C^{14}. This is in agreement with our findings.

In Figure 9, log of the rate of excretion of conjugated steroids in micrograms per minute has been plotted against time. The half-life is estimated to be four hours, a value in agreement with that of Hellman and his associates for the half-life of free and conjugated combined. It should be emphasized that we measured steroids with a 17,21-dihydroxy-20-ketone group, whereas Hellman and his associates measured all steroids with C^{14} in ring A.

COMMENT

The adrenal cortex plays an important role in the resistance of the organism to environmental change. The patient with Addison's disease or the adrenalectomized animal succumbs to any one of a great variety of stresses which the intact animal can readily resist. Furthermore, these same stressful stimuli excite the pituitary adrenocortical system of an intact organism to increase the production of cortical steroids. The important question arises as to why the organism requires supplementary quantities of adrenocorticosteroids to manifest its full capacities to survive a noxious stimulus. The resistance of the animal to certain stressful stimuli may be explained in terms of known metabolic actions of the cortical steroids. However, the mechanism of resistance to the great majority of noxious stimuli remains unexplained. Furthermore, we are unable to answer the question as to whether the functional actions of the adrenocorticosteroids are coupled with a chemical transformation in these substances. Sayers, in a review (10) of this problem, was inclined to the view that adrenocorticosteroids are "utilized" at an accelerated rate during stress. However, that he was aware of the inadequacy of the experimental evidence for such a concept is borne out by his statement that "whether degradation is a necessary accompaniment of steroid hormone action or is an entirely unrelated and incidental phenomenon is a problem of fundamental importance. Until such time as these points are clarified it is well to apply the word 'utilization' of cortical steroids without any implication as to the relation between degradation and functional activity of the hormone." If by "utilization of adrenocorticosteroids" is meant a multitude of processes including excretion, degradation, and concentration in certain tissues, which act to reduce the concentration of these substances in the blood, then the gross metabolic analyses of the present report do not support the thesis that stress increases the rate of "utilization" of adrenocorticosteroids. There was no significant difference between the plasma level of hydrocortisone at the end of infusion of this steroid in the insufficiency test when the patients were in crisis and the plasma level in the test applied at a time when the patient appeared to be in a state of eucorticism. Furthermore, the rate of decline in steroid level in the plasma subsequent to the end of infusion appeared to be the same in all tests. The experiments suggest that severe adrenocortical insufficiency is associated with a renal impairment in the excretion of steroids. However, it is doubtful that this impairment significantly altered the over-all "utilization" of hydrocortisone.

Studies designed to measure rate of disappearance of hydrocortisone from blood and rate of appearance of degradation products in urine, as exemplified by the work of Hellman and his coworkers and the work reported here, do not offer support for the thesis that adrenocorticosteroids are "utilized" at an accelerated rate during stress. However, they do not represent evidence against such a thesis. Strictly degradative processes may go on at a much faster rate than conversions which are coupled with the functional activity of the adrenocorticosteroids. The influence of such conversions on the over-all rate of disappearance of hydrocortisone from the body fluids may be masked by the strictly degradative processes. Both *in vivo* (8, 11, 12) and *in vitro* (13–15) experiments indicate that the liver plays a major role in the disappearance of hydrocortisone from the blood. That the chemical transformations in the liver are not essential for the biological activity of hydrocortisone is suggested by the fact that this steroid can exhibit its metabolic actions in the hepatectomized animal (16). Whether degradation in the liver plus excretion by the kidney can account for all of the hydrocortisone which disappears has not been determined. Until such time as this point is clarified, the concept that stress increases tissue "utilization" of adrenocorticosteroids must be considered with reservation

as the explanation for the accelerated production of adrenocorticosteroids which accompanies application of a stressful stimulus.

SUMMARY AND CONCLUSIONS

Hydrocortisone disappears rapidly from the vascular compartment following its intravenous administration. At the end of a 30-minute period of infusion of 100 mg. of hydrocortisone only 4 mg. remained in the total plasma volume. The half-life of the steroid, the time for the concentration of plasma hydrocortisone at the end of the infusion to be reduced to one-half, was estimated to be one and one-third to two and one-third hours.

The rate of disappearance of hydrocortisone from the plasma as measured under the conditions of these experiments appears to be uninfluenced by the state of the tissues in regard to adrenocortical hormone. The results do not support the thesis that hydrocortisone disappears more rapidly from the blood in adrenocortical insufficiency as compared to eucorticism. However, gross over-all metabolic studies of this type do not disprove this thesis.

The renal excretion of free steroids with a 17,21-dihydroxy-20-ketone group parallels the plasma concentration of free hydrocortisone. Conjugation to glucuronides occurs rapidly; glucuronides of steroids with a 17,21-dihydroxy-20-ketone group were excreted during the first 15 minutes of the infusion. The peak rate of excretion of glucuronides usually occurred in the 30-minute period following the end of infusion. Subsequently, the rate of excretion remained elevated for three hours and then declined to reach a low level after eight hours.

The major fraction of the free steroids was excreted during the first four hours after infusion of hydrocortisone; the major fraction of the conjugated steroids was excreted during the first eight hours. The free steroids excreted during the 24 hours following infusion accounted for approximately 4 per cent of the administered hydrocortisone; the conjugated steroids accounted for approximately 20 per cent.

The results suggest that renal excretion of free and of conjugated 17,21-dihydroxy-20-ketones is impaired in patients with Addison's disease who have had no previous steroid therapy.

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FACTORS INFLUENCING THE COURSE OF MERCURIAL DIURESIS DURING PITRESSIN® INFUSION IN NORMAL SUBJECTS [1, 2]

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Studies on the mechanism and site of mercurial action have led to conflicting conclusions, because of differences in experimental design. Thus, since sodium excretion during heavy sodium loading did not exceed about 20 per cent of that filtered, Duggan and Pitts (1) deduced that the action of mercurials is exerted upon the distal segment, although additional effects on the proximal tubules could not be excluded. On the other hand, Farah, Cobbey, and Mook (2) obtained sodium excretions under mercurials approximating 35 to 40 per cent of that filtered, a significantly greater fraction than that generally attributed to the distal tubule.

In an earlier study (3), the hypertonic urine resulting from continuous infusion of concentrated sodium chloride solution was found to be diluted almost to isotonicity during mercurial diuresis. Since facultative water reabsorption, under control of the anti-diuretic hormone, occurs low in the nephron and is probably unaffected by mercurials (4), it was suggested that this decrease in concentration may reflect inhibition of more proximal isosmotic reabsorption. From the changes in urine osmolarity, Brodsky and Graubarth (5), who studied hydropenic animals, and Welt, Goodyer, Darragh, Abele, and Meroney (6) who studied three men receiving Pitressin® infusions, drew similar conclusions. More recently, Capps, Wiggins, Axelrod, and Pitts (7) and Dale and Sanderson (8) suggested that because Mersalyl increased sodium and chloride excretion without augmenting urine volume during maximal water diuresis, mercury and post-pituitary ADH must act on the same (distal) segment. Subsequently, however, the latter investigators (9) indicated uncertainty as to the renal site of mercurial diuretic effect. Studies on renal excretion of substances other than sodium and chloride have demonstrated that mercurials inhibit certain proximal (10, 11), but not other, presumably distal, tubular functions (3).

The present report is concerned with an extension of our earlier observations (3) on the dilution of hypertonic urines following administration of mercurials during infusion of a concentrated salt solution. Because the resulting increasing electrolyte excretion complicates analysis of the data, constant and virtually maximal distal reabsorption of water was maintained by the continuous infusion of Pitressin® throughout the procedure.

MATERIAL AND METHODS

The eleven subjects, all of whom were free of clinically evident cardiovascular and renal disease, were previously maintained on regular diets. Each study was begun between 8 and 9 A.M., with the patient in the postabsorptive state. Hydration was maintained by means of a measured oral water intake and an intravenous infusion of physiological saline or lactated Ringer's solution given at 3 to 4 ml. per minute by means of a constant infusion pump. Following several control collection periods, Pitressin® (1 to 3 mu. per Kg.) was injected intravenously, and a quantity calculated to provide an equivalent dose each 60 or 90 minutes was added to the infusion mixture.[4] Fairly constant anti-diuresis was thereby achieved although, at times, slowly increasing concentration of urine occurred.

When constant minimal urine flow was achieved, 2 ml. (80 mg. Hg) of mercaptomerin, a xanthine-free mercurial diuretic, were injected intravenously, and fre-

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[4] One patient, L. G., received 6 mu. per Kg. with consequent excessive Pitressin® effect.

TABLE I

Procedure (1)	Time (2)	Urine: Flow (3)	Urine: Sodium Conc. (4)	Urine: Sodium Exc. (5)	Urine: Chloride Conc. (6)	Urine: Chloride Exc. (7)	Urine: Potassium Conc. (8)	Urine: Potassium Exc. (9)	Urine: Osmolarity* Conc. (10)	Urine: Osmolarity* Exc. (11)	"Tubular" rejectate (post-mercurial): Water (12)	Sodium Exc. (13)	Sodium Conc. (14)	Chloride Exc. (15)	Chloride Conc. (16)	Osmolarity Exc. (17)	Osmolarity Conc. (18)	Serum: Osmol. (19)	Serum: Na (20)	Serum: Cl (21)	Serum: K (22)
	min.	*ml./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mOs./L.*	*μOs./min.*	*ml./min.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μOs./min.*	*mOs./L.*	*mOs./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
J. L., Age 50; 65 Kg., Hepatitis, Conv. 1/6/51																					
Begin inf.†	14	10.5	7	76	10	110	5.6	59	26	270									135	96.8	4.53
	12½	11.0	7	81	11	123	5.9	65	27	293											
	17	11.7	8	91	9	105	5.7	67	27	316											
	13½	11.9	9	103	10	117	5.1	61	28	329									136	98.0	4.73
Pit. 1 mu./Kg.‡	13	3.1	25	78	31	98	18.2	58	86	271											
	20	1.0	115	120	130	137	75.5	79	381	400											
	20	1.0	124	124	125	125	51.3	51	350	350									135	97.5	4.48
	25	1.1	155	167	134	145	47.1	51	404	437											
Hg§	25	1.0	172	166	148	142	42.6	41	429	412											
	30	1.3	195	260	168	223	22.0	29	434	578	0.3	93	300	78	252	141	455		136	97.7	4.67
	20	4.6	158	725	159	731	6.0	28	327	1,500	3.6	558	156	586	164	1,060	296				
	20	6.6	151	1,000	151	1,000	4.1	27	311	2,070	5.6	833	148	858	152	1,630	289				
	20	7.2	150	1,080	150	1,080	3.0	21	306	2,200	6.2	841	136	937	151	1,760	285				
	14	8.2	149	1,230	152	1,250	2.5	21	304	2,500	7.2	1,060	148	1,100	153	2,060	286		134	96.4	4.66
	15	7.2	155	1,110	156	1,120	3.4	25	317	2,280	6.2	948	153	975	158	1,840	288				
	15½	5.8	166	966	166	964	3.3	19	340	1,970	4.8	799	167	819	171	1,530	320				
	20	5.8	163	948	161	932	2.9	17	333	1,930	4.8	781	163	787	165	1,490	312		133	95.9	4.66
T. S., Age 50, 70.4 Kg., Pulmonary fibrosis, Bronchiectasis 3/20/51																					
Begin inf.	17½	1.0	141	145	180	185	74.0	76	430	443											
	23½	1.9	69	131	91	174	38.1	73	214	409											
	15	4.2	31	132	39	165	16.9	71	96	408											
	15	5.9	24	141	28	167	12.1	71	72	426											
	25	7.5	22	167	26	195	10.0	75	65	486											
Pit. 2 mu./Kg.	18	2.6	122	323	124	327	36.3	96	317	837											
	20	1.5	225	344	223	342	54.2	83	558	854											
	45½	1.2	227	280	228	280	43.1	53	540	664											
	27½	1.0	220	224	219	223	57.4	58	555	566											
Hg	22½	1.1	202	220	208	227	45.5	50	495	540	0.1			4	572						
	19	4.3	180	779	185	797	10.9	47	382	1,650	3.3	555	168	574	174	796	241				
	14½	8.5	159	1,360	164	1,400	7.1	61	332	2,840	7.5	1,130	151	1,180	157	1,990	264				
	15	11.0	154	1,690	157	1,720	6.2	68	320	3,520	10.0	1,460	147	1,500	150	2,670	267				
	19	8.2	163	1,340	165	1,360	6.7	55	339	2,790	7.2	1,110	155	1,140	158	1,940	269				
	12½	7.8	171	1,340	174	1,370	7.0	55	356	2,790	6.8	1,110	164	1,150	168	1,940	284				
	17½	7.3	175	1,270	179	1,300	6.8	49	364	2,640	6.2	1,040	168	1,070	172	1,790	286				

* Osmolarity calculated, except in patients P. F., P. G., and C. W.
† Begin constant (3.4 or 3.0 ml. per min.) intravenous infusion of NaCl (in patients J. L., T. S., E. M.) or Lactated Ringers' (in patients P. F., P. G., C. W.) solution.
‡ Dose given stat., and equivalent dose per 60 minutes placed into infusion.
§ Thiomerin®—2 ml., I.V.

TABLE I—*Continued*

Procedure (1)	Time (2)	Urine: Flow (3)	Urine: Sodium Conc. (4)	Urine: Sodium Exc. (5)	Urine: Chloride Conc. (6)	Urine: Chloride Exc. (7)	Urine: Potassium Conc. (8)	Urine: Potassium Exc. (9)	Urine: Osmolarity* Conc. (10)	Urine: Osmolarity* Exc. (11)	"Tubular" rejectate (post-mercurial): Water (12)	Sodium Exc. (13)	Sodium Conc. (14)	Chloride Exc. (15)	Chloride Conc. (16)	Osmolarity Exc. (17)	Osmolarity Conc. (18)	Serum: Osmol. (19)	Serum: Na (20)	Serum: Cl (21)	Serum: K (22)
	min.	*ml./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mOs./L.*	*μOs./min.*	*ml./min.*	*μEq./min.*	*mEq./L.*	*μEq./min.*	*mEq./L.*	*μOs./min.*	*mOs./L.*	*mOs./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
E. M., Age 39, 59.1 Kg., Hyperthyroidism 3/27/51																					
Begin inf.	12½	12.5	20	248	20	250	8.3	104	56	703									142	104	4.67
	13½	10.3	25	263	26	272	8.3	86	68	696											
	15½	12.6	21	271	22	276	6.2	78	55	698											
	18	14.3	18	262	20	281	5.7	82	48	687									141	104	4.63
Pit. 3 mu./Kg.	16	3.9	58	224	54	209	17.9	69	151	586											
	39½	1.2	203	239	215	254	41.5	49	489	578									140	104	4.25
	32	1.1	226	258	231	264	27.4	31	507	578											
	39½	1.0	246	254	242	249	30.2	31	552	569									138	103	4.07
	22	1.0	255	255	256	256	29.0	29	568	568											
Hg	17	1.1	240	255	240	255	26.7	28	533	565									138	103	4.07
	18	2.4	196	467	204	489	13.4	32	419	1,000	1.4	212	152	233	168	432	311				
	10	11.7	156	1,820	160	1,870	3.6	42	319	3,730	10.7	1,570	147	1,610	151	3,160	295				
	11½	15.4	155	2,390	153	2,360	2.8	44	316	4,870	14.4	2,130	148	2,100	146	4,300	299				
	11	15.2	155	2,350	154	2,340	2.6	39	315	4,790	14.2	2,090	148	2,080	147	4,220	297		136	99	4.17
	12	14.2	155	2,190	154	2,190	2.5	35	315	4,490	13.2	1,930	146	1,930	146	3,920	296				
	13	12.7	162	2,060	162	2,060	2.6	33	329	4,180	11.7	1,800	154	1,800	154	3,610	309				
	16	10.4	165	1,720	164	1,710	2.8	29	336	3,500	9.4	1,460	156	1,450	155	2,930	312		135	100	4.11
P. F., Age 19, 70 Kg., Acute rheumatic fever—Healed 5/19/53																					
Begin inf.	49	1.3	270	340	289	364	96.4	122	873	1,100											
	10	4.7	32	152	42	198	4.75	175	210	998								290	140	105	1.82
	9	8.1	23	191	28	227	22.9	186	144	1,170								292	141	104	3.87
	8	5.7	33	187	38	216	26.8	154	174	1,000											
	10	4.4	50	222	55	245	21.3	95	235	1,050											
Pit. 3 mu./Kg.	24	1.4	122	168	136	188	32.8	45	516	712								292	141	105	3.80
	36	1.1	174	197	177	200	69.2	78	714	807											
	37	1.1	217	232	233	249	56.0	60	761	814											
	26	1.2	223	274	201	247	57.9	71	772	949								294	142	105	3.80
	23	1.1	254	277	227	247	51.3	56	818	892											
	19	1.3	246	310	207	261	44.0	55	735	926											
	22	1.0	268	273	220	224	39.4	40	832	849								296	143	103	3.60
Hg	15	0.9	265	240	220	200	40.0	36	804	729											
	17	0.9	275	252	217	199	41.2	38	819	752											
	18	1.4	225	315	218	305	36.0	50	687	962	0.4	42	111	81	213	113	297				
	10	7.9	160	1,260	162	1,280	7.3	58	385	3,040	6.9	992	144	1,050	154	2,190	319	296	143	104	3.58
	7	13.1	153	2,000	147	1,930	3.8	50	350	4,590	12.0	1,720	143	1,700	141	3,740	310				
	8	17.4	152	2,650	170	2,960	2.7	46	340	5,920	16.3	2,370	145	2,730	167	5,070	310				
	8	18.5	151	2,790	149	2,760	2.1	39	330	6,100	17.4	2,510	144	2,530	145	5,250	300				
	8	17.6	155	2,730	152	2,680	2.1	37	334	5,880	16.5	2,450	148	2,450	148	5,030	303	292	141	103	3.75
	8	15.5	157	2,430	155	2,400	2.2	34	346	5,360	14.4	2,150	149	2,170	150	4,510	312				
	8	16.2	158	2,570	159	2,580	2.0	32	348	5,660	15.2	2,290	151	2,350	155	4,810	316				
	12	13.5	159	2,150	158	2,130	2.3	31	358	4,830	12.4	1,870	150	1,900	153	3,980	319				
	11	11.8	165	1,950	166	1,960	3.1	37	363	4,290	10.7	1,670	156	1,730	161	3,440	319				
	22	11.2	165	1,850	167	1,870	3.0	34	364	4,080	10.1	1,570	155	1,640	162	3,230	318	292	141	101	3.78
	22	11.5	164	1,890	160	1,840	3.6	41	367	4,220	10.4	1,610	154	1,610	154	3,370	321				
	21	10.3	165	1,700	166	1,710	4.7	48	369	3,800	9.3	1,420	154	1,480	160	2,950	318	286	138	100	3.82

TABLE I—*Continued*

Procedure (1)	Time (2)	Urine: Flow (3)	Urine: Sodium Conc. (4)	Urine: Sodium Exc. (5)	Urine: Chloride Conc. (6)	Urine: Chloride Exc. (7)	Urine: Potassium Conc. (8)	Urine: Potassium Exc. (9)	Urine: Osmolarity* Conc. (10)	Urine: Osmolarity* Exc. (11)	"Tubular" rejectate (post-mercurial): Water (12)	"Tubular" rejectate: Sodium Exc. (13)	"Tubular" rejectate: Sodium Conc. (14)	"Tubular" rejectate: Chloride Exc. (15)	"Tubular" rejectate: Chloride Conc. (16)	"Tubular" rejectate: Osmolarity Exc. (17)	"Tubular" rejectate: Osmolarity Conc. (18)	Serum: Osmol. (19)	Serum: Na (20)	Serum: Cl (21)	Serum: K (22)
	min.	ml./min.	mEq./L.	μEq./min.	mEq./L.	μEq./min.	mEq./L.	μEq./min.	mOs./L.	μOs./min.	ml./min.	μEq./min.	mEq./L.	μEq./min.	mEq./L.	μOs./min.	mOs./L.	mOs./L.	mEq./L.	mEq./L.	mEq./L.
P. G., Age 23, 56 Kg., Disseminated sclerosis 6/23/53																					
Begin inf.	13	6.8	14	95	13	92	9.2	63	83												
	9½	10.9	17	187	9	102	8.0	88	75									284	142	105	4.13
	10	10.6	17	180	9	94	7.6	80	72												
	12½	6.6	23	150	17	114	10.3	68	102												
Pit. 3 mu./Kg.	30	0.7	151	113	116	87	72.3	54	615	461								284	142	104	4.04
	20	0.7	189	134	133	94	64.4	46	744	528											
	20	0.7	224	150	144	96	57.2	38	795	533											
	20	0.7	246	182	155	115	58.8	43	830	614											
Hg	20	0.8	260	203	153	119	55.4	43	820	640								278	140	103	4.13
	21	0.8	278	236	177	150	46.0	39	821	696	0.1	54	391	33	239	56	405				
	19	1.1	254	275	202	219	30.6	33	755	816	.4	93	249	102	276	176	470				
	10½	2.4	236	586	206	511	16.0	40	582	1,440	1.8	404	228	394	213	803	453	277	139	102	4.27
	9½	3.4	226	762	210	708	10.4	35	553	1,860	2.7	580	218	591	222	1,220	459				
	10	4.2	233	978	213	894	8.7	37	516	2,170	3.5	796	228	777	223	1,530	438				
	10	3.6	248	905	222	810	8.7	32	544	1,980	2.9	723	246	693	236	1,350	459	281	140	101	4.07
	10	3.5	259	906	232	812	10.4	36	561	1,960	2.8	721	260	695	249	1,320	473				
	10	3.4	260	892	237	812	10.1	35	575	1,970	2.7	710	261	695	255	1,330	489				
	20	3.1	274	849	244	756	12.6	39	603	1,870	2.4	662	279	639	267	1,230	514				
	20	2.7	280	750	250	670	16.3	44	640	1,710	2.0	568	288	553	281	1,080	548	270	138	103	4.12
	21½	2.3	282	640	256		19.0	43	675	1,530	1.6	458	294			892	572				
	20	2.0	294	588	257		23.2	46			1.3	406	298					270	139	100	3.96
C. W., Age 32, 76.3 Kg., Rheumatoid arthritis 12/16/54																					
Begin inf.	13	0.8	246		250													285	140	105	4.80
	24	0.9	200	192	145	139	68.0	65	783	750											
	22	1.0	207	216	155	162	45.2	47	720	752											
	23	1.1	214	228	165	176	42.0	45	720	767											
	24	1.2	216	270	172	215	39.8	50	709	886											
	18	1.2	233	284	191	233	39.3	48	739	902											
	17	1.3	240	314	194	254	37.6	49	727	952								283	143	106	4.75
Hg	20	1.2	238	295	190	236	31.6	39	716	888											
	10	1.6	220	352	187	299	16.0	26	617	987	0.3	54	159	55	162	60	176				
	9	4.3	181	774	169	723	6.1	26	426	1,820	3.0	476	157	479	158	896	297				
	9	7.8	169	1,310	159	1,240	4.0	31	367	2,860	6.5	1,010	156	996	153	1,930	297				
	8	10.8	160	1,730	157	1,700	2.8	30	345	3,730	9.5	1,430	150	1,450	152	2,800	294				
	8	12.9	160	2,060	156	2,010	2.1	28	332	4,280	11.6	1,760	151	1,760	152	3,350	289				
	8	14.2	158	2,310	156	2,220	1.6	23	331	4,720	12.9	2,010	155	1,970	152	3,790	292				
	8	14.0	164	2,310	159	2,240	2.0	28	333	4,680	12.8	2,010	157	1,990	156	3,750	293				
	8	13.2	167	2,210	161	2,130	1.8	24	340	4,510	11.9	1,910	159	1,880	157	3,580	299	278	137	104	4.56
	10	13.2	169	2,240	162	2,150	1.8	24	339	4,490	11.9	1,940	162	1,900	159	3,560	297				
	12	11.9	169	2,010	163	1,940	2.1	25	343	4,090	10.6	1,710	160	1,690	159	3,160	297				
	15	9.8	166	1,630	165	1,620	4.0	39	356	3,490	8.5	1,330	156	1,370	161	2,560	300				

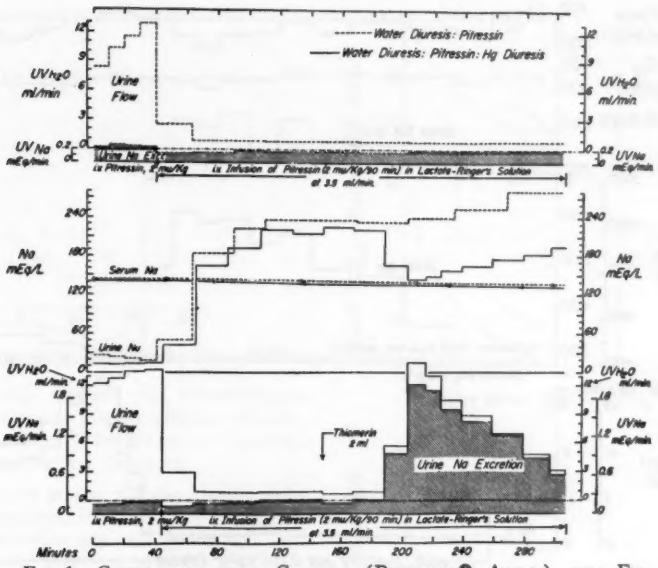


FIG. 1. COMPARISON OF A CONTROL (PITRESSIN® ALONE) AND EXPERIMENTAL (PITRESSIN® PLUS MERCURIAL) PROCEDURE IN THE SAME SUBJECT (M. S.)

The urine flow and sodium excretion during the control study are depicted in the upper third of the graph (broken line). In the middle third, the control serum and urinary sodium concentrations (broken lines) are plotted together with those of the experimental study (solid lines). Note the decline in urinary sodium concentration toward isotonicity associated with the increasing urine volume and sodium excretion (solid lines below) during mercurial diuresis.

quent urine collections were continued until the resulting diuresis had ebbed significantly.

Blood was removed periodically from an indwelling arterial needle. Urine specimens were collected by washing the bladder with 10 or 15 ml. of sterile distilled water and 100 to 200 ml. of air through an indwelling, multi-holed, rubber catheter. When osmolarity was measured, air alone was used for emptying the bladder.

The determinations of inulin, para-aminohippurate, sodium, chloride, and potassium, in plasma and urine, were performed by the standard methods employed in this laboratory (3). Total solute concentration was measured in some patients with a thermistor-osmometer.[5]

RESULTS AND DISCUSSION

The results are summarized in Table I and Figures 1–7. In those patients studied prior to the availability of the osmometer, the urinary electrolyte osmolarity was calculated as: $2([Na] + [K])$, ignoring the contribution of urea and other substances of low molecular weight.

[5] Manufactured by Fiske Associates, Inc., Boston, Mass.

Effects of Pitressin® infusion on electrolyte and water excretion

Administration of Pitressin® produced a rapid fall in urine flow and a corresponding rise in urinary solute concentrations. In two control studies (Figure 1) in which the Pitressin® infusion was continued for several hours and the mercurial withheld, the urine flow and concentration of sodium and chloride remained constant, independent of the usual diurnal variation.

In all but one subject (T. S.), Pitressin® produced an initial decrease in electrolyte output. This fall is probably factitious in that the decreased urine flow results in a greater relative quantity of solute in the anatomical "dead space." This explanation is supported by the fact that renal hemodynamics similarly fell, and together with urinary electrolyte excretion, invariably rose again during the very next period. Following this transient fall, the urinary sodium and chloride

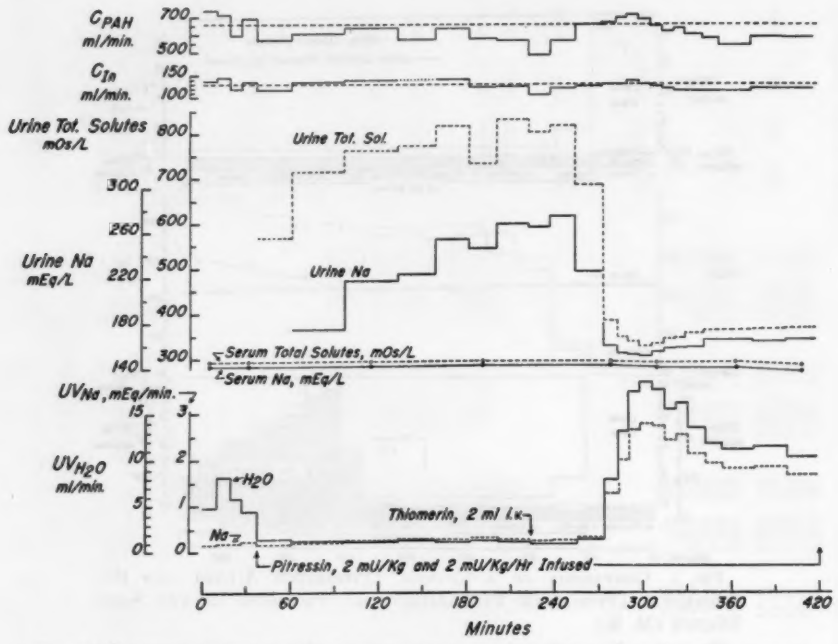


FIG. 2. RESPONSE OF SUBJECT P. F. TO ADMINISTRATION OF MERCURIAL DIURETIC DURING PITRESSIN® ADMINISTRATION

Note that with maximal diuresis, the urinary sodium and total solute concentrations most closely approach isotonicity. Renal hemodynamics do not change significantly during the procedure.

excretion usually rose, reaching or slightly exceeding the control levels. Whereas initial chloride concentration generally exceeded that of sodium, following Pitressin® administration, the sodium concentration tended to equal or slightly exceed that of chloride in three patients (J. L., R. C., T. S.), none of whom received excessive sodium as PAH. The natriuretic action of Pitressin®, postulated by some (12) may explain this increased urinary Na/Cl ratio. The fall in urinary excretion of potassium, despite apparently insignificant changes in its serum level may reflect, in part, the post-absorptive state.

Throughout the procedure, serum electrolyte concentration usually fell slightly, probably secondary to dilution, and tended to rise again during diuresis.

Effect of mercurial diuresis on urinary electrolytes during Pitressin® infusion

On the doses of Pitressin® and mercurial administered, a clear-cut diuretic response to the mercurial was obtained in each case. Following the usual latent period, urine flow increased to maximal values of 4.2 to 18.5 ml. per min., and gradually subsided.[6] This increase in urine flow and salt excretion was invariably accompanied by a fall in urinary sodium and chloride concentrations which, following maximal diuresis, rose toward the previous, more hypertonic levels. The potassium excretion continued to fall in these normal individuals in striking contrast to the increase in potassium excretion following mercurials observed in cardiac patients or other subjects exhibiting increased sodium conservation.

Examination of Table I and Figures 1 and 2 reveals that the lowest urinary concentration of sodium and chloride attained during diuresis always exceeded the patient's plasma sodium level. Moreover, the greater the increase in urine flow, the more closely did the urinary sodium concentration approach isotonicity (Figure 3). These data

[6] The procedures had to be discontinued before return to the original control levels was reached.

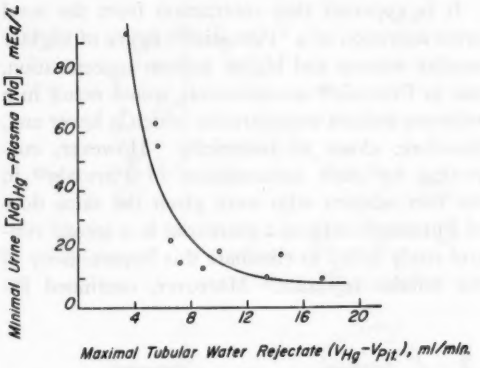


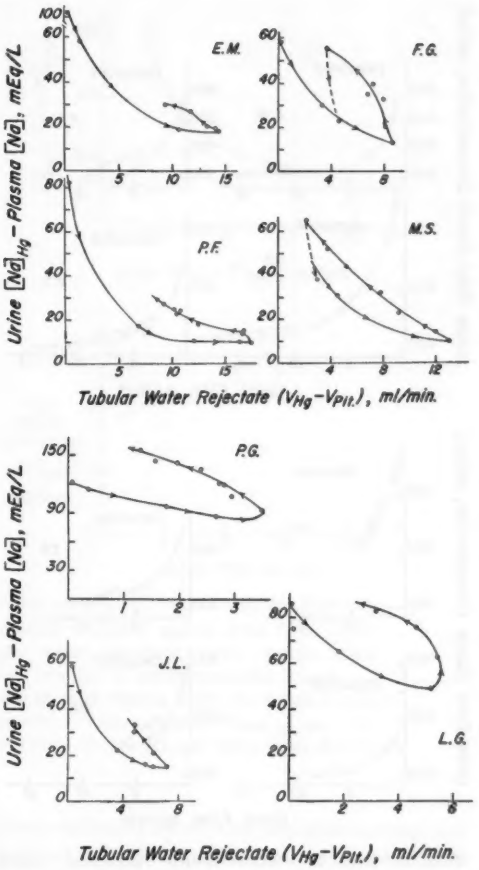
FIG. 3. RELATIONSHIP BETWEEN THE URINE-PLASMA CONCENTRATION DIFFERENCE (URINE $[Na]_{Hg}$ − PLASMA [Na]) AND TUBULAR WATER REJECTATE AT MAXIMAL DIURESIS ($V_{Hg} - V_{Plt}$) IN ALL SUBJECTS

It is apparent that the greater the diuresis, especially beyond 4 to 6 ml. per min., the more closely does the urinary sodium concentration approach isotonicity. This strongly suggests that the hypertonic urine excreted under Pitressin® alone is diluted by an approximately isotonic fluid.

support the hypothesis that the very small volume of highly concentrated urine resulting from Pitressin® administration is diluted by an increasingly larger volume of isotonic fluid, rejected by the tubule due to the mercurial's action. In such case, no matter how great the diuresis, the final concentration must, however slightly, exceed isotonicity.

The data in Columns 14, 16, and 18 reveal that, following administration of the mercurial, the calculated tubular rejectate concentrations [7] of solutes (osmolarity) or chloride in the tubular fluid rejected under the influence of the mercurial may be determined (Table I, Columns 16 and 18).

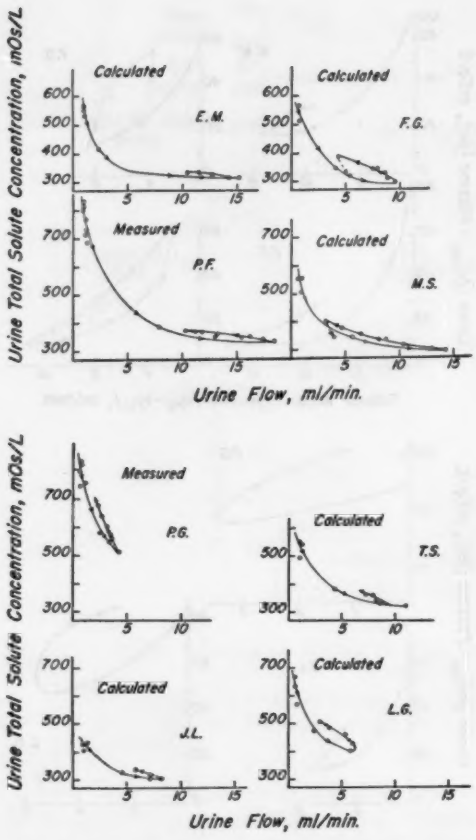
[7] The tubular rejectate concentrations are calculated as follows: If the constant flow under Pitressin® alone is subtracted from the total urine flow during mercurial diuresis, the difference represents the additional water passing down, and unresorbed by, the distal tubules, working almost maximally. This quantity has been designated the tubular water rejectate (Table I, Column 12, Figures 3 and 4). Similarly, if the sodium excreted under Pitressin® alone, just prior to the administration of the mercurial, is subtracted from that during mercurial diuresis, the difference represents the additional sodium now reaching, and unresorbed by, the distal segment (*i.e.*, the tubular sodium rejectate, Table I, Column 13). By dividing this tubular sodium rejectate by the tubular water rejectate, the concentration of sodium in the additional urine resulting from the mercurial's effect may be calculated (Table I, Column 14). Similarly, the



FIGS. 4a AND 4b. RELATIONSHIP BETWEEN THE URINE-PLASMA SODIUM CONCENTRATION DIFFERENCE (URINE $[Na]_{Hg}$ − PLASMA [Na]) AND THE TUBULAR WATER REJECTATE ($V_{Hg} - V_{Plt}$) DURING THE COURSE OF DIURESIS IN INDIVIDUAL SUBJECTS

During the phase of increasing urine flow, urinary sodium concentration falls toward that of plasma.

During falling urine flow, each curve returns at a somewhat higher level, indicating a greater concentration of electrolyte at any given flow. Moreover, the smaller the diuresis, the greater is this deviation. These curves suggest that additional resorption of a relatively constant volume of water occurs during waning flows. The arrows indicate course of diuresis. The broken line portions of the curves for subjects F. G. and M. S. were obtained following discontinuance of Pitressin®.

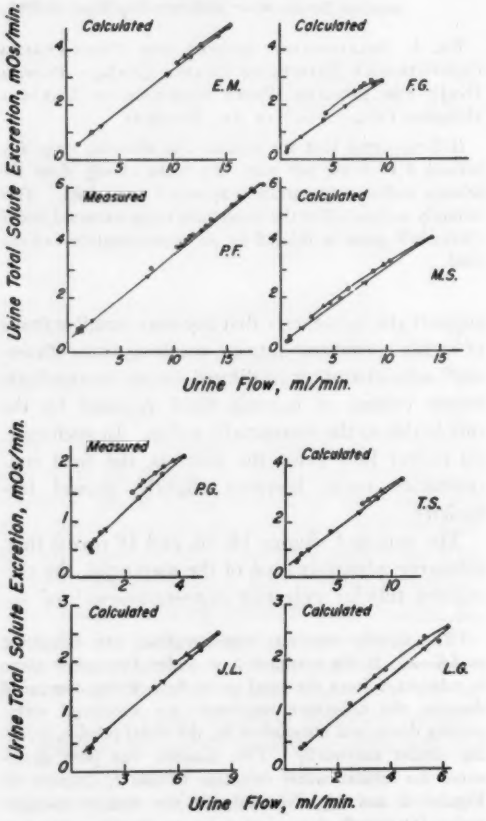


FIGS. 5a AND 5b. RELATIONSHIP BETWEEN URINE TOTAL SOLUTE CONCENTRATION AND URINE FLOW DURING THE COURSE OF DIURESIS IN INDIVIDUAL SUBJECTS

The deviations in the curves during waning diuresis are similar to those observed in Figures 4a and 4b. Total solute concentrations were calculated as $2([Na]+[K])$ in most subjects and measured in two (P. F., P. G.).

dium, chloride, and total solutes are not isotonic, but approach a limiting value somewhat above the plasma level. One explanation for this might lie in the fact that a steady state of anti-diuresis may not have been achieved prior to the administration of the mercurial. Thus, in two subjects (R. F., T. S.), the urine flow was still falling slightly at the time the mercurial was given. Moreover, in the two control studies, the urine solute concentrations continued to rise slowly as the Pitressin® infusions were maintained.

It is apparent that subtraction from the total urine excretion of a "Pitressin®" figure of slightly smaller volume and higher sodium concentration, due to Pitressin® accumulation, would result in a rejectate sodium concentration which is lower and, therefore, closer to isotonicity. However, correcting for such accumulation of Pitressin® in the two subjects who were given the same dose of Pitressin® without a mercurial in a second control study failed to eliminate this hypertonicity of the tubular rejectate. Moreover, continued Pi-



FIGS. 6a AND 6b. RELATIONSHIP BETWEEN URINE TOTAL SOLUTE EXCRETION AND URINE FLOW DURING THE COURSE OF DIURESIS IN INDIVIDUAL SUBJECTS

The linear relationship and the general similarity of slope are consistent with the postulated dilution of the originally hypertonic urine by an approximately isotonic fluid. Note the smaller urine flow per mOs. excreted during waning diuresis.

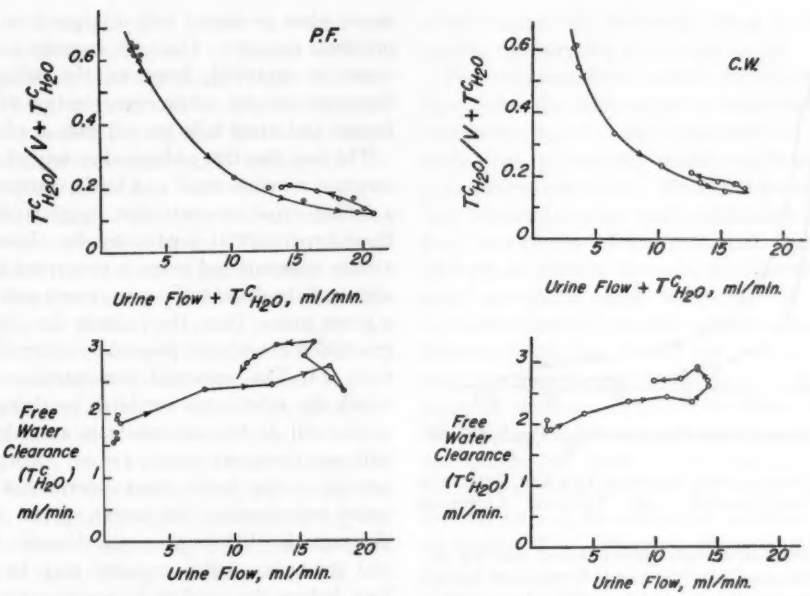


FIG. 7. CHANGES IN NEGATIVE FREE WATER CLEARANCE DURING THE COURSE OF DIURESIS

In the lower portion, the negative free water clearance is plotted against urine flow. With the onset of diuresis, there is an increase in tubular water resorption, which remains fairly constant until maximal flow. Subsequently, a further increase in water resorption occurs.

The upper portion demonstrates that as the volume of fluid passing down the tubule (urine flow (V) + $T^c_{H_2O}$) increases during diuresis, the fraction of water resorbed $T^c_{H_2O}/(T^c_{H_2O}+V)$ anisosmotically falls. As diuresis subsides, this reabsorbed fraction at any given urine flow is increased.

tressin® accumulation would lead to rising rejectate sodium concentrations during increasing diuresis. Actually the converse occurs, the tubular rejectate sodium concentration being maximal at the onset of diuresis, and falling as the urine flow increases.

Other factors may contribute to the hypertonicity of the tubular rejectate with regard to sodium. These include the fall in potassium excretion during most experiments and the isosmotic reabsorption of other constituents of the glomerular filtrate, such as glucose. It is of interest that during osmotic (urea or mannitol) diuresis, complete reabsorption of glucose (13) contributes 5 mM per L. to the isosmotic resorbate concentration.[8] However, a real increase in rejectate tonicity is indicated by the fact that this increase was observed not only when total solute concentrations were calculated, but also when measured directly by osmometry.

The persistence of a slightly hypertonic tubular rejectate suggests that during mercurial diuresis a small additional volume of water is resorbed from an isotonic tubular fluid, presumably distal to the site of mercurial action. At the onset of diuresis, when the volume of fluid passing down from the proximal segment has increased only slightly, this will result in a significant increase in the calculated rejectate sodium concentration. However, as the tubular flow increases, the distal

[8] During forced osmotic diuresis, relatively small amounts of the diuretic agent are reabsorbed. Therefore, the sodium concentration of the isosmotic reabsorbate is greater, and that of the rejectate less, than that of the glomerular filtrate. Conversely, mercurial diuretics which exert a primary effect on sodium reabsorption, would, assuming the resorbate to be isosmotic, produce a tubular rejectate hypertonic with respect to sodium, the degree of hypertonicity reflecting the contribution of other substances to the isotonicity of the resorbate.

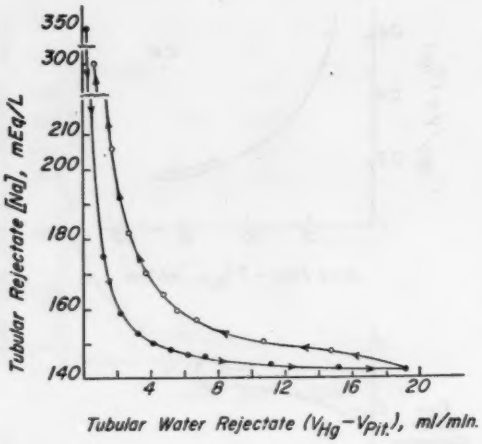


FIG. 8. RELATIONSHIP BETWEEN TUBULAR REJECTATE SODIUM CONCENTRATION AND TUBULAR REJECTATE VOLUME

This hypothetical diuresis curve is based upon the following assumptions: At the onset (Pitressin® alone), the urine flow is 1.0 ml. per min., and urinary sodium concentration is 288 mEq. per L. During increasing diuresis, a constant increment of 0.3 ml. per min. of water without solute is absorbed; following maximal flow, an additional 0.5 ml. per min. of water is reabsorbed. It is apparent that at smaller urine flows, additional reabsorption of a given amount of water without solute will produce a considerably greater increase in tubular rejectate sodium concentration than at higher flows.

tubular capacity to reabsorb water at this Pitressin® dose is soon exceeded, and the small increment of water resorption assumes progressively less importance. Subsequently, the additional rejectate remains isotonic and continues to dilute the concentration of both the urine (Figure 5) and the tubular rejectate (Figure 8).

This explanation is compatible with the findings of an initially high tubular rejectate sodium or solute concentration which asymptotically approaches isotonic levels, as urine flow increases, and rises again, as urine flow falls. The theoretical additional distal water resorption required to produce the observed rejectate sodium concentrations is very small in comparison with the total distal water resorption of 15 to 20 ml. per min. probably occurring under the conditions of our experiments (Figure 8). It appears, then, that the distal tubules, already absorbing water at a near-maximal rate can reabsorb some additional water when presented with a larger flow from the proximal segment. Once the capacity to reabsorb water is exceeded, however, the urine volume increases and the solute concentration of both rejectate and urine falls toward that of plasma.

The fact that this additional moiety of water resorption remains small and fairly constant despite a falling urine concentration suggests that under these experimental conditions, the elaboration of a more concentrated urine is prevented by the inability of the distal tubules to resorb water beyond a given rate. Thus, the capacity for distal water resorption is evidently limited by either of two factors: 1) The maximal concentration gradient which the tubule can establish by doing osmotic work; and 2) the maximal rate at which the tubule can transport water, *per se*. Normally, the osmotic ceiling is the chief determinant of distal water reabsorption, but under special conditions like osmotic (13) or mercurial diuresis, the maximal water resorptive capacity may be exceeded long before the predicted concentration gradient for a given level of anti-diuretic activity is reached.

If this additional distal water absorption were due to Pitressin® accumulation, the (negative) free water clearance [9] would rise throughout the procedure. However, examination of Figure 7 reveals that the negative free water clearance re-

[9] The free water clearance ($T^c_{H_2O}$) as defined by Wesson and Anslow (14), is that amount of water which must be added to or removed from urine to render it isosmotic with the plasma. $T^c_{H_2O} = [1 - (U_{osm}/P_{osm})]V$, where U_{osm} and P_{osm} are the osmolarities of the urine and plasma, respectively, and V is the urine flow. $T^c_{H_2O}$ is negative when the urine is hyper-osmotic, and positive when hypo-osmotic. It should be noted that the Pitressin®-induced formation of a hypertonic urine from an isotonic filtrate is not necessarily equated with the entire distal tubule, either in an anatomical or in the usual physiological sense. These investigators subdivided distal tubular reabsorption of water into at least two components: 1) Facultative, which is associated with active distal sodium reabsorption ($T^d_{H_2O}$), leaving the urine isotonic; and 2) hyper-osmotic, which concentrates the urine lower in the distal tubules or in the collecting ducts ($T^c_{H_2O}$). Of these, only the latter represents the free water clearance. Ladd (15) and Zak, Brun, and Smith (16) have demonstrated that mercurials do not affect the $T^c_{H_2O}$. Whatever water diuresis occurs following a mercurial must therefore be due to the osmotic effect of the increased solutes claiming excretion or to a direct effect upon water reabsorption proximal to the segment where concentration occurs.

mains fairly constant prior to the mercurial and rises only as diuresis begins. In accord with the above hypothesis, once the distal tubular capacity for reabsorbing water at this level of anti-diuretic activity is exceeded, the negative free water clearance remains at this new value throughout the period of rising urine flow.

Following maximal diuresis, the negative free water clearance increases again. The significance of this second rise becomes apparent from examination of Figures 4 to 6, which reveals that during falling urine flows, the curves invariably fail to trace the path followed during the diuretic phase. If mercurial diuresis represents a relatively simple process which rises to maximal intensity and subsides as the pharmacologic effect is dissipated, then the curves would be expected to rise and fall symmetrically; that is, along the same path. Instead, in each instance, during the falling phase, the urine, at any given flow, is more concentrated than during increasing diuresis.

When the rise and fall in urine flow during diuresis are plotted against sodium or total solute excretion (Figure 6), in some patients an almost linear relationship appears to exist. However, the return to low urinary flows during the falling phase occurs at a slightly higher rate of sodium or total solute excretion per volume of urine. It would appear that following maximal diuresis, the kidney reabsorbs more water than previously, for less water is excreted per mOsm. of solute as diuresis subsides.

Since it is unlikely that the mercurial directly caused this increased tubular water reabsorption, and since significant Pitressin® accumulation did not occur during these studies, the possibility that mercurial diuresis may have evoked other mechanisms affecting water excretion must be considered. Previous studies from this laboratory (17) have shown that a large fraction of intravenously administered mercury appears in the urine before diuresis has begun. More significantly, as much as ¼ to ⅓ of the injected mercury is excreted after diuresis has subsided. Therefore, although little is known of the relationship between the chemical form and diuretic activity of mercury, the fall in urine flow cannot be ascribed simply to the absence of mercury as such. Rather, it may reflect secondary activation of compensatory mechanisms which combat the mercurial effect by increasing tubular reabsorption of salt and water.

The relationship of solute excretion to urine flow under the influence of a mercurial has been studied by Brodsky and Graubarth (5) in hydropenic dogs, presumably under conditions of continued release of ADH. From the linear relationship between osmotic load (excretion) and urine flow, they concluded that mercurial diuresis is essentially an osmotic diuresis, the amount of distal water resorption being determined by the number rather than the chemical nature of the particles claiming excretion. While the present data support this concept, the altered level of the waning phase of diuresis suggests that other factors or mechanisms may modify this relationship. Thus, the amount of distal water reabsorption, and therefore of osmotic work performed, is a function not only of the number of particles involved, as suggested by Rapoport, Brodsky, West, and Mackler (18), but of the degree of anti-diuretic activity at the time. Only during maximal antidiuresis does the simpler relationship apply.

That the increased sodium and calculated total solute concentrations during the falling urine flows do not merely reflect decreased concentration of other solute particles, for example, urea, is established by the finding of similar changes in measured urinary osmolarity. Further evidence that increased reabsorption of water occurred following maximal mercurial action is provided by the calculated free water clearances ($T^c_{H_2O}$) (Figure 7). Because the concentration of urine tends to approximate that of plasma during mercurial diuresis, mercurials produce no marked effects on the free water clearance, after the initial increase discussed above. However, as diuresis subsides, the (negative) free water clearance at any given urine flow is higher than during the periods of increasing flow (Figure 7), indicating that, following maximal diuresis, more water in excess of solute is being reabsorbed. Similarly, the ratio of free water clearance ($T^c_{H_2O}$) to the total fluid leaving the proximal or isosmotic segment (urine volume + $T^c_{H_2O}$), or the fraction of water of the isotonic mixture absorbed distally, falls, as expected, during increasing diuresis, and returns at a higher level as flow subsides. Thus, a greater fraction of water per unit urine flow down the distal tubules is removed from the iso-

tonic mixture during the waning phase of diuresis. Because the rise in $T^c_{H_2O}$ is stepwise rather than continuous, these data do not simply reflect accumulation of Pitressin® during the procedure, but suggest instead that some other, presumably endogenous, mechanism is activated.

Of interest is the finding of a tubular rejectate chloride concentration approximating that of sodium. While it has been contended, on the basis of the potentiating action of chloruretic agents, that mercurials act primarily to inhibit chloride resorption (19), our data neither support nor refute this hypothesis. The tubular rejectate chloride concentration of about 140 mEq. per L., confirming the results of the micropuncture experiments of Walker, Bott, Oliver, and MacDowell (20), reflects rather the combined influences of the Donnan effect, as a result of which the glomerular filtrate chloride concentration may reach 112 to 115 mEq. per L., and the virtually complete, isotonic reabsorption of bicarbonate, phosphate, and glucose (13, 14). The high chloride concentration in the tubular rejectate and urine is simply the result of the normal plasma ion partition, and does not help localize the site of mercurial action.

The decreased urinary potassium excretion during mercurial diuresis in these normal subjects is in direct contrast to the increased potassium excretion observed in individuals in whom sodium conserving mechanisms have been activated by low sodium intake, specific cardiac, hepatic, or renal diseases, adrenal cortical steroid administration, or ammonium chloride acidosis. Potassium excretion involves filtration at the glomerulus, proximal reabsorption, and distal secretion of this cation. A purely proximal tubular inhibition by mercurials should result in increased potassium excretion in both normal and cardiac subjects. An effect limited to the distal segment, if it inhibited potassium secretion, would account for the decreased excretion encountered in normal subjects, but not for the increased excretion of potassium in cardiacs and other sodium retainers.

The explanation for this particular effect of the mercurial may depend upon the balance of factors adjusting potassium excretion at a given time to the body's previous conditioning. Thus, in normal, post-absorptive subjects, any potassium escaping reabsorption more proximally ordinarily would be largely reabsorbed lower in the tubule. In subjects exhibiting marked sodium conservation, the increased sodium delivered to the distal tubule as a result of mercurial inhibition of proximal reabsorption, is exchanged for potassium, ammonia, and hydrogen ion, by distal tubular base-conserving mechanisms, which are unaffected by therapeutic doses of mercurial (3). This leads to both an increased excretion of potassium and the relatively greater excretion of chloride than sodium (21, 22).

On the basis of available evidence, certain tentative conclusions as to the site and mode of action of mercurial diuretics in man may be drawn. The failure of mercurials to increase urine flow during maximal water diuresis suggests a primary effect on the distal tubule salt reabsorption (7, 8). However, the following observations strongly suggest a predominant diuretic effect on the proximal segments: 1) Minimal toxic doses of mercury produce histologic damage and histochemical evidence of enzyme inhibition only in cells of proximal tubules; 2) mercurials depress renal transport of substances other than sodium and chloride, presumably handled by the proximal segments (10, 11) but not those secreted by the distal tubule (3); and 3) the changes in hydrogen ion and potassium excretion following mercurials in man (3, 21). The present data and analogous studies from other laboratories support such an interpretation.

The degree and duration of mercurial diuresis ultimately depend upon the balance between factors increasing or decreasing water and electrolyte reabsorption by the tubules. The present data suggest that, with the reduction in extracellular fluid volume resulting from diuresis, mechanisms normally preserving body fluid volume are activated, leading to increased reabsorption of electrolyte and water. In addition to explaining the described alterations in rejectate solute concentration, this hypothesis is consistent with other facts regarding mercurial diuresis. For example, despite their normal renal hemodynamics, normal serum electrolytes, and high urinary sodium and chloride excretion rates, normal subjects on regular diets exhibit a shorter, and smaller, diuretic response to mercurials than do edematous patients on low sodium diets, who are actively re-

taining sodium. The normal subject achieves a peak diuretic response greater than that of the cardiac, but, with the consequent decrease in extracellular fluid and plasma volume (23), sooner invokes secondary mechanisms for maintaining body fluid volume and the circulation. Further studies on these mechanisms which result in increased tubular reabsorption of sodium and water are now in progress.

SUMMARY AND CONCLUSIONS

1. Mercurial administration, in hydrated patients receiving 1 to 3 mu. per Kg. per hr. of Pitressin® intravenously and excreting a hypertonic urine of low volume (0.6 to 1.0 ml. per min.), results in a diuresis of urine slightly exceeding isotonicity. This implies that the effect of the mercurial is to add a relatively large volume of isotonic urine to the previously small, hypertonic flow. Support for this explanation is found in the fact that the greater the diuresis the more closely is plasma tonicity approached.

2. The fact that mercurial diuresis is isotonic implies that the principal site of action is proximal to the segment of the nephron at which anisosmotic or free water resorption occurs.

3. The calculated concentration of sodium in the fluid unresorbed (rejected) by the tubules as a result of mercurial action is hypertonic to that of plasma. The probable reasons for this phenomenon have been discussed.

4. The relationship between urinary total solute concentration, or excretion, and urine flow during the rising and subsiding phases of mercurial diuresis strongly suggests that an altered bodily or renal response results in somewhat greater reabsorption of water per unit load of solutes excreted after maximal diuresis has been attained. Calculation of the free water clearance corroborates this finding.

5. It is suggested that the subsidence of mercurial diuresis represents not simply the dissipation of a drug effect, but, in part, reflects the activation of compensatory counter-mechanisms promoting fluid retention.

ACKNOWLEDGMENT

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THE EFFECTS OF EXCLUSION OF THE RIGHT VENTRICLE FROM THE CIRCULATION IN DOGS[1]

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In experiments bearing on the relationship of "backward failure" of the right ventricle to the elevated venous pressure of clinical heart failure, Starr, Jeffers, and Meade (1) found that extensive cauterization of the right ventricle in dogs was not followed by a significant elevation of venous pressure. Subsequent investigations of the effects of destructive procedures on the right ventricular myocardium have led further to the conclusion that actively functioning right ventricular muscle is not necessary for the maintenance of adequate blood flow through the low pressure pulmonary circulation (2, 3).

Rodbard and Wagner (4) created right atrio-pulmonary artery anastomoses in dogs and occluded right ventricular outflow. Blood was observed to flow directly from the right auricle to the pulmonary artery, and these animals survived up to one hour. Despite these short survivals, and without quantitatively documenting the alterations produced by this acute procedure, these authors concluded that the venous pressure may drive venous return through the lungs without benefit of the right ventricular pump.

If it is possible to by-pass the right ventricle, such observations have clinical as well as basic physiologic importance. However, experiments in progress (5) suggest an active role by the right ventricle in the regulation and maintenance of a normal peripheral venous pressure and adequate pulmonary blood flow. Therefore, it was decided to re-investigate the veno-pulmonary anastomoses briefly studied by Rodbard and Wagner (4).

MATERIALS AND METHODS

Freeze-dried heterografts of either pig or calf aorta were prepared in the manner described by Hufnagel, Rabil, and Reed (6) and used as veno-pulmonary anastomoses in each of three ways (Figure 1). Group I, consisting of four dogs, had grafts placed between the right auricle and main pulmonary artery. In these animals, attempts were made to occlude the root of the pulmonary artery acutely so that blood gained access to the pulmonary circulation only through the shunt.

Group II, consisting of two dogs, had shunts placed between the superior vena cava and the main pulmonary artery. After freeing the posterior right auricular wall from the adherent right pulmonary veins, a non-crushing clamp was placed across the right auricle in such a manner that the inferior and superior venae cavae were in communication with each other and the shunt, no blood entering the right ventricle (Figure 1).

Group III, thirteen dogs, had shunts placed from both venae cavae to the main pulmonary artery in the manner illustrated (Figure 1). In these dogs, both venae cavae were acutely occluded at their auricular orifices.

Healthy mongrel dogs, weighing 9 to 16 Kg. were anesthetized with sodium pentobarbital, 25 mgm. per Kg. intravenously, and maintained on positive pressure respirations with 100 per cent oxygen via endotracheal tube. Following the usual surgical preparation, the chest was opened with a generous transverse sternal-splitting incision in the 7th intercostal space.

The pulmonary artery was dissected completely free after partial incision of the pericardium. (When possible, the heart was kept within the pericardium, except in Group I animals when marked right ventricular dilatation was imminent.) The azygos vein was ligated in each procedure.

Partial occlusion for end-to-side anastomoses to the great vessels was accomplished with acutely angled and curved Potts non-crushing clamps. Anastomoses were made with a 5–0 silk suture on atraumatic needles. The auricular anastomoses in Group I animals was made to the stump of the amputated appendage. In Group III animals, it was found best to place the pulmonary artery and inferior vena cava anastomoses first. The pulmonary artery graft was then anastomosed to the superior vena cava. Finally the inferior vena caval limb of the shunt was connected to the pulmonary artery-superior vena

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[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

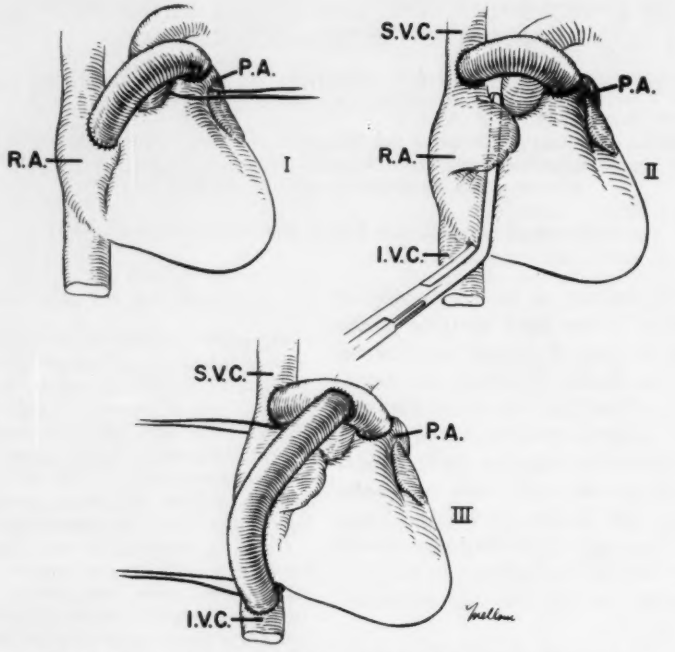

FIG. 1. DIAGRAMS OF THREE TYPES OF VENO-PULMONARY SHUNTS
Ligatures and clamp represent points of occlusion of normal pathway of blood in acute experiments. See text for discussion.

cava limb to form a "T" as shown in Figure 1. All grafts were at least as large in internal diameter as the vessels to which they were anastomosed, care being taken to provide wide orifices for unimpeded blood flow.

The operation occasionally lasted up to 3 hours in Group III animals, but frequently only one hour in Group I. Acute studies were performed at the end of this period only in those animals with efficient circulations, as evidenced by the absence of hypotension, slowing of the heart rate or cardiac dilatation.

Intravascular pressures were recorded by strain-gage transducers [3] and carrier-wave type amplifiers on a direct-writing multiple channel oscillograph.[4] Pressures were obtained by threading short lengths of 2.5 mm. I.D. polyethylene tubing into the aorta through an iliac artery and into the inferior vena cava through an iliac vein. Pulmonary artery and shunt pressures were obtained in the open chest animals by inserting 1 mm. I.D. polyethylene catheters, or short length of 7F ureteral catheters directly into these vessels through purse-string sutures.

Shunt pressures in each group were measured near the pulmonary artery anastomosis. In Figures 3 and 4, prior to the instant designated "Shunt open," the shunts were occluded with non-crushing clamps, and the pressures recorded were from the segments in direct communication with the pulmonary artery. The zero baseline for shunt pressure tracings was at the level of the right auricle.

All experiments were monitored with continuous electrocardiographic recordings. Flow measurements were attempted using the indicator-dilution curve technique; these generally were unsuccessful because of low and unsteady flow rates during the acute studies.

The material used for infusion in acute studies was a 5 per cent gelatin solution in isotonic sodium chloride (Plazmoid®) [5] which was warmed to 37° C. prior to intravenous administration.

Two animals in Group I, one in Group II and one in Group III were permitted to survive for from 2 to 6 weeks with shunts in place but without pulmonary artery, right auricle or venae cavae occlusions. In these animals, angiocardiograms were made, using the Sanchez-Perez Automatic Seriograph.[6] Roentgenograms were taken at one-half second intervals. Urokon® sodium, 70 per cent, was injected directly into the great veins and right heart through polyethylene catheters.

[3] Statham Laboratories, Beverly Hills, California.

[4] Sanborn Company, Cambridge, Massachusetts.

[5] Plazmoid® was generously supplied by Dr. Joseph P. Webb, Upjohn Company, Kalamazoo, Michigan.

[6] Automatic Seriograph Corporation, College Park, Maryland.

TABLE I

The effects on intravascular pressures of acute occlusion of right ventricular outflow and inflow in Group I and Group II animals, respectively

(Each value noted is the mean for the number of acute occlusions shown. Occlusion values were taken at or near the time of maximal inferior vena cava pressure rise.)

Dog	Number of acute occlusions	Mean duration of occlusion (*seconds*)	Pre-occlusion and occlusion values: Mean aortic pressure (*mm. Hg*) Pre-	Post-	Mean P.A. pressure (*mm. Hg*) Pre-	Post-	Shunt pressure (*mm. Hg*) Pre-	Post-	I.V.C. pressure (*mm. Hg*) Pre-	Post-	Heart rate (*beats/min.*) Pre-	Post-
IA	6	46	77	17	12	6.5			4.0	6.5	170	170
IB	3	20	95	21	24	8.0			9.0	12.5	170	185
IC	3	49	70	22			2.0	3.0	4.0	7.0	140	140
ID	2	29	112	22	7.0	3.0			*6.0	11.0	170	170
IIA	4	64	81	26			2.3	3.6	6.0	9.0	150	120
IIB	3	72	78	15			2.0	4.2	6.5	12.0	* 110	110

* Right auricular pressure.

RESULTS

Angiocardiograms. In preparations of each group studied from 2 to 6 weeks postoperatively with shunts in place but without right ventricular inflow or outflow occlusions, angiocardiograms revealed flow from pulmonary artery to right auricle or venae cavae in accordance with the normal pressure gradient.

Group I: Acute occlusion of right ventricular outflow

The hemodynamic alterations following acute occlusion of the pulmonary artery in four Group I animals are summarized in Table I, and a typical example is shown in Figure 2. In each of the fourteen acute experiments, a uniformly reproducible sequence of events occurred. After occlusion, the inferior vena caval pressure rose and the aortic and pulmonary arterial pressure fell precipitously. The right ventricle dilated markedly, and "ventricularization" of the venous pressure pulse appeared indicating marked tricuspid insufficiency. (Tricuspid insufficiency in this experimental situation was also noted by Rodbard and Wagner [4].) The shunt became dilated, filling from the auricular side, although evidently flow from auricle to pulmonary artery was meager. A pressure pulse appeared in the pulmonary artery which was of ventricular origin and transmitted via the regurgitant tricuspid valve, auricle and shunt.

The occlusion was held until it was evident that cardiac contraction had nearly ceased. This varied from 18 to 78 seconds. The occlusion of 78 sec-

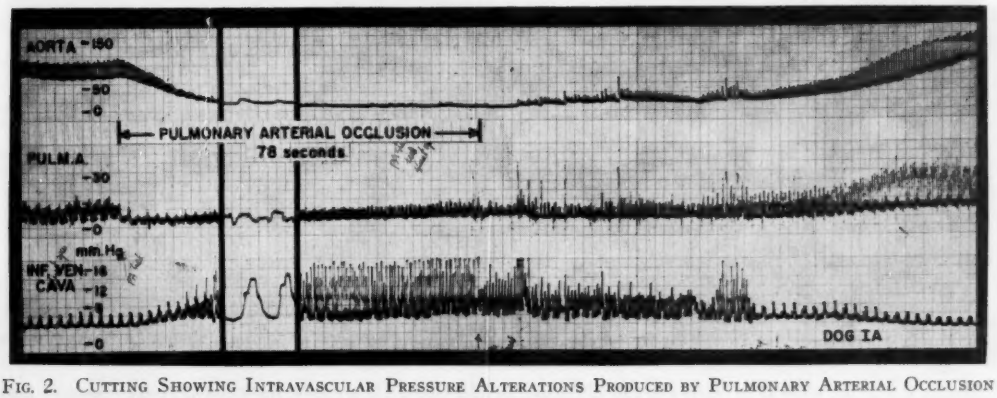

FIG. 2. CUTTING SHOWING INTRAVASCULAR PRESSURE ALTERATIONS PRODUCED BY PULMONARY ARTERIAL OCCLUSION IN DOG IA

Slow paper speed was 2.5 mm. per sec., fast speed 25 mm. per sec. See text for discussion.

onds shown in Figure 2 was the longest held in this group and it was evident that aortic blood flow had all but ceased. Prompt recovery usually followed release of the pulmonary artery and no attempts were made to measure the duration of life during pulmonary arterial occlusion.

The enormous dilatation of the right ventricle in this experiment possibly could have caused embarrassment of *left* ventricular function by displacement of the interventricular septum. Therefore, Shunt II was devised in order to eliminate this factor.

Group II: Acute occlusion of right ventricular inflow

Data from these seven acute experiments in two dogs are shown in Table I. Since acute dilatation of the right ventricle was eliminated, it was possible to hold right auricular occlusions for longer periods than in the Group I experiments. Figure 3 depicts the most favorable response observed to right auricular occlusion which was held in this instance for 164 seconds. The pressure pulse in the shunt was of auricular origin, probably arising in the unclamped portion of the auricle. After complete recovery in this animal, the right auricular clamp was re-applied. Cardiac function declined gradually with bradycardia and by 64 seconds there was a markedly diminished aortic pulsation. This dog lapsed into ventricular fibrillation during chest closure.

It was evident that although right ventricular dilatation was eliminated in this type of shunt, the experiment still did not permit a critical analysis of the factors under study. The operation was technically difficult, requiring careful dissection of the posterior right auricular wall and right pulmonary veins. Despite the lack of conduction abnormalities as indicated by the electrocardiogram, this experiment carried the risk of damaging the sino-atrial conduction apparatus. Finally, it was possible to occlude the coronary sinus unknowingly. Therefore, Shunt III was devised.

Group III: Acute occlusion of both venae cavae at their auricular orifices

Table II and Figure 4 indicate the responses of Group III animals to venae caval (and azygos vein) occlusion. The response consistently observed was again a marked fall in mean systemic

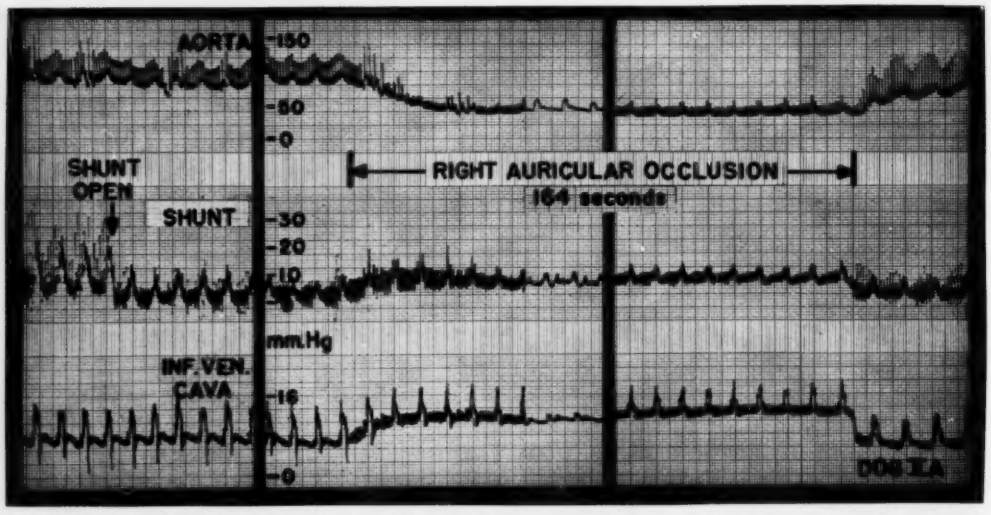

FIG. 3. CUTTING SHOWING INTRAVASCULAR PRESSURE ALTERATIONS PRODUCED BY RIGHT AURICULAR OCCLUSION IN DOG IIA

Paper speeds the same as in Figure 2. In all Group II and Group III animals, the shunt pressure measured near the pulmonary artery showed a wide pulse pressure prior to opening the shunt. When the shunt was opened, this pulse pressure narrowed as flow towards the vena cava was established. See text for further discussion.

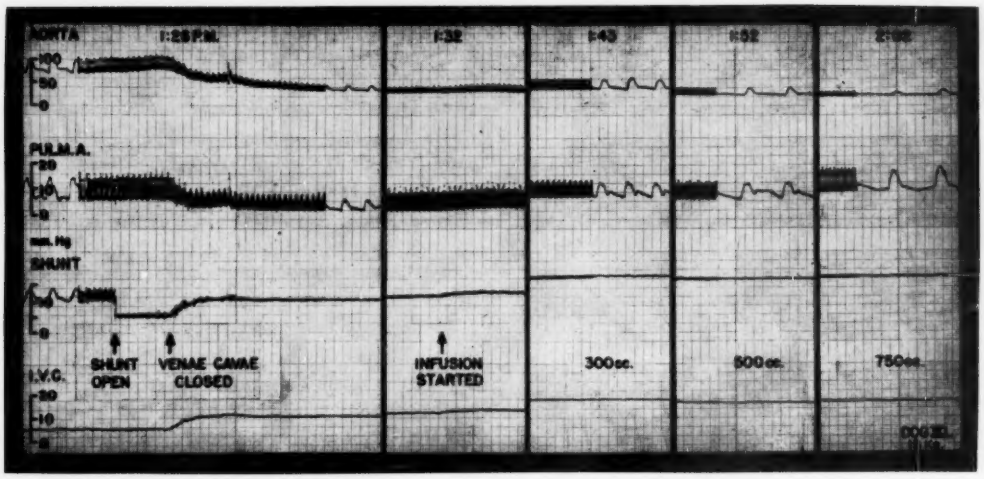

FIG. 4. CUTTING SHOWING INTRAVASCULAR PRESSURE ALTERATIONS PRODUCED BY VENAE CAVAL OCCLUSION IN DOG IIIL AND RESPONSE TO INTRAVENOUS INFUSION

Slow paper speed 1.0 mm. per sec., fast speed 25 mm. per sec. See text and Table II for explanation.

arterial pressure (to the range of 20 to 36 mm. Hg) accompanied by a fall in pulmonary arterial pressure. At the same time, both shunt and inferior vena caval pressures rose, the latter rising to levels between 9 and 22 mm. Hg. The right ventricle did not dilate. The pressure pulse in the pulmonary artery was probably due to ejection of coronary sinus blood through the competent pulmonic valve. Without infusions, these animals exhibited a steady decline in circulatory volume with progressive bradycardia and hypotension despite markedly elevated central venous pressures. In three animals the total duration of circulatory function was between 6 and 12 minutes at the end of which time the heart had ceased beating.

Survival was prolonged in Group III animals by the infusion of gelatin solution administered intravenously at rates of 20 to 25 cc. per minute (Table II, Figure 4). When the infusion began inferior vena caval and shunt pressures rose promptly. Mean aortic pressure rose, the average maximum elevation being 11 mm. Hg. At the time of maximal response in aortic pressure, central venous pressures ranged from 15 to 30 mm. Hg representing a mean rise of 9 mm. Hg. Figure 5 shows the means of the pressure alterations occurring in the four infusion experiments and em-

TABLE II

Alterations produced by permanent occlusion of both venae cavae (and azygos vein) in Group III dogs

(Post-occlusion values were taken at the time of maximal rise in inferior vena cava pressure, post-infusion values at time of maximal response in aortic pressure.)

	Pre- and post-occlusion values								Max. response to infusion				Total vol. infusion at max. response	Duration circulatory function
	Mean aortic pressure (*mm. Hg*)		Mean P.A. pressure (*mm. Hg*)		Shunt pressure (*mm. Hg*)		I.V.C. pressure (*mm. Hg*)		M.aor. press. (*mm. Hg*)	M.P.A. press. (*mm. Hg*)	Shunt press. (*mm. Hg*)	I.V.C. press. (*mm. Hg*)	(*cc./Kg. B.W.*)	(*minutes*)
Dog	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-						
IIIA	68	20	10.0	6.0			8.0	18.0						12
IIIC	80	32	12.0	5.0			7.0	13.0						6
IIIE	82	36	14.0	12.0			8.0	22.0						7
IIIB	85	31			8.0	10.0	8.0	17.0	40		25.0	30.0	32	50
IIIJ	60	20	9.0	4.0			7.0	14.0	30	8.0		15.0	10	13
IIIK	75	30	10.0	4.0	6.0	6.5	5.0	9.0	45	7.5	11.0	15.0	15	50
IIIL	90	32	8.0	3.0	6.0	11.0	6.0	11.5	43	9.0	18.0	19.0	24	39

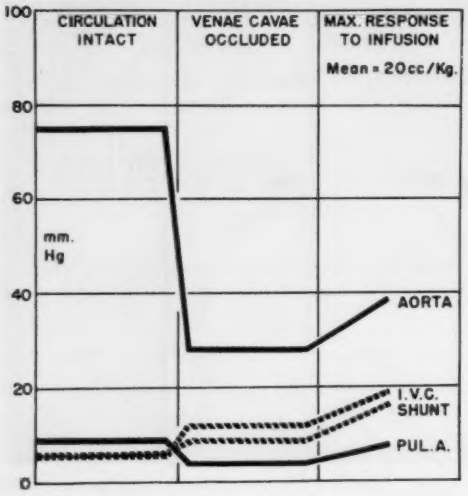

FIG. 5. CHART OF MEAN INTRAVASCULAR PRESSURES IN FOUR GROUP III ANIMALS IN RESPONSE TO VENAE CAVAL OCCLUSION AND INTRAVENOUS INFUSION

Note gradient in pressure established from aorta to pulmonary artery.

phasizes the pressure gradient established from aorta to pulmonary artery when the right ventricle was by-passed.

Attempts to rule out reflex depression of aortic pressure following venae caval occlusion were carried out in five additional Group III animals. Two animals were given blocking doses of hexamethonium intravenously prior to occlusion. The course of occlusion was unaltered and systemic pressures were extremely low (18 and 12 mm. Hg). These animals survived 5 and 12 minutes, respectively. In the remaining three Group III animals the pulmonary artery was occluded at the same time as the venae caval occlusion. These occlusions were maintained for brief periods up to 30 seconds, during which time the right ventricle was kept full. There were no significant differences noted between occlusion of venae cavae alone and occlusion of venae cavae plus pulmonary artery, except that the small pulse in the pulmonary artery was obliterated by pulmonary artery occlusion. It was concluded that hypotensive reflexes resulting from the lack of normal atrial or ventricular distension were probably of no significance in these experiments.

DISCUSSION

It appears from the experiments described that the central venous pressure cannot force an adequate blood flow through the lungs in an intact animal without benefit of the right ventricular pump. Despite the low resistance offered by the pulmonary circulation, the loss of pressure from aorta to pulmonary artery (Figure 5) is too great for maintenance of the total circulation without the added energy supplied by right ventricular contraction.

It should be emphasized that the data presented were obtained in acute experiments. Observations were made only of the effects of acute and total occlusion of right ventricular inflow or outflow. The possibility exists that gradual closure of the normal pathway of blood through the right ventricle in these preparations might yield somewhat different results.

Jamison, Gemeinhardt, Alai, and Bailey (7) have given support to the relative "unimportance" of the right ventricle in maintenance of the total circulation with experiments in which a pump was used to replace the left ventricle in dogs. They found that when ventricular fibrillation was induced, circulation through the lungs was maintained. Experiments in progress (5) using a mechanical "left ventricle" of controlled constant output (8), indicate that when "pure" right ventricular failure is produced in this manner, normal blood flows can only be maintained under conditions of hypervolemia and marked venous hypertension of a level not possible to attain in the present experiments using an intact circulation. The present investigation was undertaken because the views of Rodbard and Wagner (4) were incompatible with the observations made with the mechanical "left ventricle."

The observations of Starr, Jeffers, and Meade (1), Bakos (2) and Kagan (9) (using cautery), and Donald and Essex (3) (using right coronary artery ligation) that destruction of the right ventricular wall does not produce significant increments in venous pressure imply that these procedures do not destroy right ventricular function. In fact, a pressure pulse was demonstrated in the pulmonary artery after extensive right ventricular myocardial destruction (2, 3, 9).

Bakos (2) suggested that the left ventricle transmits its energy to the damaged right ven-

tricle through muscle bands which circumscribe both ventricles. Kagan (9) suggested that following right ventricular destruction, the pumping action of the right ventricle may be maintained by a subsidiary mechanism such as the contraction of the common interventricular septum. Thus, both of these authors ascribe the pumping action of the right ventricle to a function of the left ventricle. However, Wiggers (10) has recently provided evidence for independent performance of the right ventricle in relation to the left. The present study provides no clue to the source of the energy for contraction of a damaged right ventricle.

Finally, the studies of Landis, Brown, Fauteux, and Wise (11) in experimental right heart failure have demonstrated the importance of an intact right ventricle in the maintenance of normal venous pressure during and following exercise and infusions.

It would appear, then, from the experiments described, and from a review of previous studies, that the right ventricle is an essential pump for the maintenance of normal flows and pressures in the intact circulation. When the right ventricle is acutely excluded from the circulation, the resulting elevated venous pressure is not capable of maintaining adequate lung perfusion.

SUMMARY AND CONCLUSIONS

Studies of destructive procedures on the right ventricular myocardium have suggested that its active function is not essential for adequate pulmonary blood flow. Experiments are discussed in which veno-pulmonary shunts were used to bypass the right ventricle in dogs. Flow through the shunts was from pulmonary artery to right auricle or venae cavae in accordance with the normal pressure gradient. Acute occlusion of the normal pathway of blood through the right ventricle resulted in marked falls in systemic arterial pressure and elevations of venous pressure. However, even when the venous pressure was elevated with infusions to levels above the normal mean pulmonary arterial pressure, lung perfusion through the shunts remained inadequate.

Thus, the right ventricle is an essential pump for the maintenance of normal pressures and flows in the intact circulation. In these studies, there was no evidence that the total circulation could be maintained when the right ventricle was acutely by-passed. In previous studies, in which attempts were made to destroy the right ventricular myocardium, right ventricular function was not destroyed.

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ESTIMATION OF UTERINE BLOOD FLOW IN NORMAL HUMAN PREGNANCY AT TERM [1]

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The blood flow to the pregnant uterus is of evident importance in supplying to the fetus the raw materials required for its growth and development and in removing from the fetal environment the waste products of metabolism. Description of the uterine blood supply was largely in anatomical terms until Barcroft and co-workers measured it directly in the rabbit (1), and indirectly in the sheep (2). It has also been calculated in the sheep (3, 4) from data obtained by fetal plethysmography (5).

METHODS

I. Theory

Estimation of human uterine blood flow at term has awaited the development of methods other than those used in animals. Recently, attempts (6, 7) have been made to apply the Fick principle (8) to the study of the maternal uterine circulation. Figure 1 presents this principle. It is modified from a derivation published by Kety (9) in a discussion of cerebral blood flow. This equation is applicable to any organ if (a) the amount of some blood-borne substance X taken up (or released) in a known time interval by that organ can be determined and if (b) the concentrations of substance X in blood *entering* and blood *leaving* the organ can be determined over the same period of time.

The most familiar application of the Fick equation is in the determination of cardiac output. Here, since the blood flow to all body tissues is being studied, the oxygen consumption of the body may be used as the numerator of the equation and the difference in oxygen content between arterial and "mixed" venous blood is used as the denominator.

The ideal test substance for use in the study of an individual organ is a blood-borne metabolite, the rate of production or destruction of which by that organ is measurable and the concentration of which in blood going to that organ and in mixed venous blood leaving that organ can be determined. As an example, hepatic blood flow has been calculated from knowledge of (a) the difference in concentrations of *urea* in blood entering and blood leaving the liver and (b) the rate of formation of urea, a process limited to the liver (10).

Kety (9) has used a foreign substance, nitrous oxide gas, for determination of *cerebral* blood flow by the Fick principle. This substance is normally not present in the body and is not metabolized in body tissues. The method can be applied to the *uterus* if the amount of nitrous oxide in the uterus at the end of the experiment can be determined and if the concentrations of nitrous oxide in arterial blood supplying and venous blood draining the uterus during the experiment can be measured. For such an application the equation derived above (Figure 1) would be modified to the following:

$$\text{Uterine Blood Flow} = \frac{N_2O \text{ in uterus}}{(N_2O) \text{ artery} - (N_2O) \text{ uterine vein}} \quad \text{(Equation } I\text{)}$$

The numerator consists of the total nitrous oxide content of the uterus and its contents at the end of the period of nitrous oxide inhalation. The denominator is the integrated arteriovenous nitrous oxide difference during the period of nitrous oxide inhalation.

II. Procedure

Observations leading to an estimation of uterine blood flow have been made at elective repeat Caesarian section at term in normal multiparous women. Spinal or epidural anesthesia is used. A mixture of 15 per cent nitrous oxide, 21 per cent oxygen, and 64 per cent nitrogen is administered in an open system through an anesthesia bag and tightly fitting mask equipped with inspiratory and expiratory valves. The mask is held in place by the anesthetist and administration of the nitrous oxide mixture started exactly at experimental time zero and continued for a period of thirty minutes plus the additional time required for delivery of the baby and placenta at the end of the thirty minutes. The total period of nitrous oxide administration is usually less than 40 minutes. No maternal or fetal complications have been observed. Kety has found that administration of 15 per

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$$\text{Amount of test substance X left in organ} = \text{Amount of X brought to organ by arterial blood} - \text{Amount of X taken away from organ by venous blood}$$

Now: $\text{Amount of X brought to organ by arterial blood} = \text{Blood flow} \times (X)_{\text{Arterial}}$

$\text{Amount of X taken from organ by venous blood} = \text{Blood flow} \times (X)_{\text{Venous}}$

So: $\text{Amount of X left in organ during experiment} = \text{Blood flow} \times \left[(X)_A - (X)_V\right]$

Or: $$\text{Blood flow} = \frac{\text{Amount of X left in organ}}{\left[(X)_A - (X)_V\right]}$$

FIGURE 1

cent nitrous oxide causes no appreciable physiologic or mental effects.

Arterial samples are taken from a brachial artery through an inlying 20-gauge needle attached to a three-way stopcock manifold as described by Kety (9). Under direct vision a polyethylene catheter is threaded into a large parametrial vein (a branch of the uterine venous plexus) through a small tributary adjacent to the uterus. The catheter is inserted to the extent that several inches of it lie within the lumen of a large tributary to the uterine vein. The small tributary is ligated proximal to the point of entry of the catheter, and again around the catheter.

A sample of uterine venous blood is drawn for use as the "venous blank." Then the administration of the nitrous oxide mixture is begun. The instant of beginning administration is called "experimental time zero." From time zero and continuing for one minute the first pair of simultaneous samples is drawn from the uterine vein and brachial artery. Pairs of samples are then drawn simultaneously from artery and vein at a rate of 2 cc. every 15 seconds from the second to third, fifth to sixth, tenth to eleventh, fifteenth to sixteenth, twentieth to twenty-first, twenty-fifth to twenty-sixth, and thirtieth to thirty-first minutes of nitrous oxide administration. Each syringe is carefully labeled as to the source and the serial number of the sample it contains. Each sample is of 8 cc. volume. The blood is drawn anaerobically into oiled heparinized syringes through the three-way stopcock manifolds described by Kety, one of which samples arterial, the other venous blood. Eight pairs of samples taken over the thirty-minute period of study have been found adequate to permit the accurate plotting of the curves of nitrous oxide concentration in artery and vein.

The nitrous oxide inhalation is continued for several minutes after these samples have been obtained. During this extra period an aliquot of amniotic fluid is aspirated through the uterine wall using a blunt No. 14 needle. The uterus is then incised and the baby extracted. Before the infant takes its first breath two pairs of hemostatic clamps are placed on a segment of umbilical cord, and the cord is cut between each pair. Administration of the nitrous oxide mixture is then discontinued. Samples of umbilical arterial and umbilical venous blood are taken anaerobically by puncture of the individual vessels in this excised loop of cord. The placenta is removed and weighed.

Analyses for nitrous oxide are done in duplicate on the sixteen maternal blood samples, the amniotic fluid sample, and the samples of fetal arterial and venous blood from the umbilical cord. Kety's modification of the method of Orcutt and Waters (11) is applied, using 2 cc. samples. Since nitrous oxide is not absorbed in the analysis, its content is calculated by subtracting a blank value of inert gas obtained by analysis of blood taken before beginning the nitrous oxide administration. This blank value has been found to be identical for maternal blood, amniotic fluid and fetal blood in samples obtained at Caesarian sections performed on patients who had not received nitrous oxide. Therefore the "venous blank" is used in calculating the nitrous oxide contents of samples taken from all sources. Oxygen and carbon dioxide contents are measured by the method of Van Slyke and Neill (12) on 1 cc. samples of maternal arterial blood, uterine venous blood and arterial and venous blood from the umbilical cord.

III. Calculation of the arteriovenous nitrous oxide difference across the uterus

To determine the denominator of (Equation *1*) the method used by Kety is directly applicable. The nitrous oxide values for arterial and venous blood are plotted against time, using the midtime of the interval over which each sample was taken as the time of that sample. A typical set of curves is shown in Figure 2. From these the denominator of (Equation *1*) is obtained by the trapezoid rule.[5] This gives the arteriovenous difference of nitrous oxide over the period of administration. In the set of curves used in Figure 2 as an example, that value is 36.10 cc. of nitrous oxide per 100 cc. of blood over the thirty-two-minute period of this experiment.

[5] In practice the average arteriovenous nitrous oxide difference for each minute of the experiment is measured from the curves and these values are added to give the nitrous oxide difference for the period of nitrous oxide inhalation beginning at experimental time zero and ending when the umbilical cord is clamped.

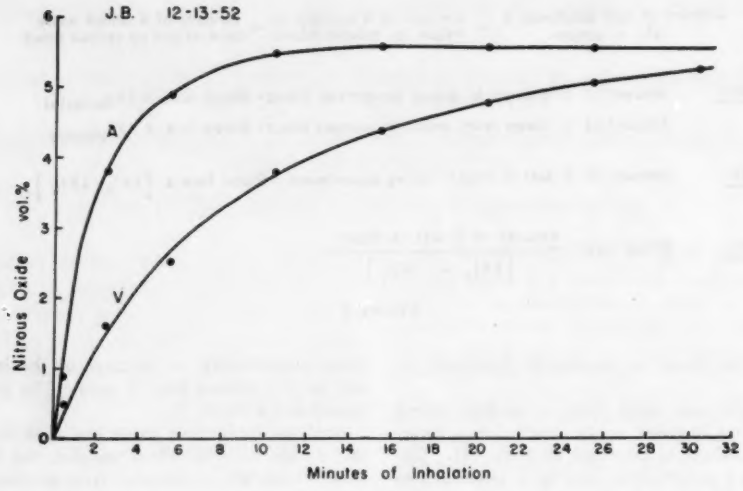

FIGURE 2

IV. Estimation of the amount of nitrous oxide in the uterus

The amount of nitrous oxide in the uterus and its contents at the end of the period of study must be determined to supply the numerator of (Equation *1*).

In the case of the brain, Kety has avoided this necessity by modifying (Equation *1*). He has found that the brain reaches nitrous oxide equilibrium with the blood draining it within a 10-minute period of nitrous oxide administration. When equilibrium is reached the concentration of nitrous oxide in the brain is the same as its concentration in the internal jugular venous blood at the time of equilibrium. With this knowledge, the equilibrium concentration of nitrous oxide in blood is substituted as the numerator of (Equation *1*). Since cerebral *concentration* of nitrous oxide is substituted for total cerebral *content* of nitrous oxide, cerebral blood flow must be expressed as cubic centimeters of blood flow per 100 gm. of brain. This modification can only be applied if equilibrium is reached during the period of nitrous oxide administration.

We have found that when nitrous oxide is administered to the pregnant woman at term Caesarian section, equilibrium between her blood and the uterine contents does *not* occur for at least one hour. There is a consistent difference between the nitrous oxide concentrations of the umbilical arterial and umbilical venous blood of the infant removed at the end of periods of nitrous oxide administration up to one hour in duration. Furthermore, although, as we have reported, (6) the solubility of nitrous oxide is identical in blood, myometrium and a homogenate of all fetal tissues,[6] even after one hour amniotic fluid does *not* reach nitrous oxide equilibrium with maternal blood. The average concentration of nitrous oxide in the amniotic fluid at the end of our experiments was 1.63 cc. of nitrous oxide per 100 cc. of amniotic fluid, as compared with an average concentration in uterine venous blood of 4.48 cc. of nitrous oxide per 100 cc. of blood. Thus the calculation of uterine blood flow on the assumption that tissue-blood equilibrium is reached in a 30-minute period of nitrous oxide administration is not acceptable. Blood flow so calculated will err in the direction of being falsely high. An alternative method has been developed as follows:

a. Experimental data available for calculation:

In our experiments the uterus is opened and its contents "sampled" at the end of the experiment. The infant and placenta are individually weighed and the concentrations of nitrous oxide are measured in the umbilical arterial and venous blood samples and the amniotic fluid sample.

b. Assumptions currently necessary:

1. That fetal and placental tissues are in nitrous oxide equilibrium with umbilical arterial blood after thirty minutes of nitrous oxide administration to the mother.

[6] The solubility of nitrous oxide in amniotic fluid and in homogenates of whole fetus and myometrium was determined by exposing these tissues to a known tension of nitrous oxide in a tonometer at 37 degrees centigrade and then analyzing the equilibrated tissues for their nitrous oxide content according to the method presented by Kety, Harmel, Broomell, and Rhode (13). The partition coefficient for nitrous oxide (the solubility of nitrous oxide in the tissue divided by the solubility of nitrous oxide in blood) was found to be 1.0 for the uterus, 0.99 for amniotic fluid and 0.96 for the whole fetus.

$$\text{Uterine Blood Flow} = \frac{N_2O \text{ in uterus}}{(N_2O)\text{ Artery} - (N_2O)\text{ Uterine Vein}} \qquad \text{(Equation 1)}$$

		Example - J.B. 12/13/52
N_2O in amniotic fluid	= Vol. amniotic fluid x N_2O in amniotic fluid	750 cc. x 1.9 cc/100 cc = 14
plus		
N_2O in fetus and placenta	= Wt. fetus + placenta x N_2O in umbilical artery	3620 gms. x 3.4 cc/100 cc = 122
plus		
N_2O in myometrium	= Wt. myometrium x N_2O in maternal artery	1000 gms. x 5.6 cc/100 cc = 56
N_2O in uterus at end of experiment		192 cc

FIGURE 3

This blood has circulated through the fetal body and is now returning to the placenta. This assumption is supported by experiments in animals, in which the fetal nitrous oxide content is directly determined and compared with the nitrous oxide concentration of umbilical arterial blood;[7]

2. That the myometrium is in nitrous oxide equilibrium with maternal arterial blood at the end of 30 minutes. This equilibrium time has been found to be adequate for myocardium and brain. We have found that the solubility of nitrous oxide in an homogenate of myometrium is identical with its solubility in blood. The maternal arterial concentration of nitrous oxide is therefore accepted as expressing the concentration of nitrous oxide in the myometrium;

3. That the average uterus weighs 1.0 Kg. at term pregnancy. This figure was taken from Reynolds (14);

4. That the average volume of amniotic fluid at term is 750 cc. The volume of amniotic fluid varies within wide limits. However, in this study the error so created is relatively small because of the low nitrous oxide content of amniotic fluid. Examination of the experiment cited below as an example will show that variation in assumed amniotic fluid volume from 100 cc. to 1,500 cc. will make an ultimate difference in calculated uterine blood flow of only 70 cc. per minute;

5. That the specific gravity of the fetus, placenta, and myometrium is equal to the specific gravity of the maternal blood. The error of this assumption is negligible.

With the facts at our disposal and the assumptions listed above, the calculations shown in Figure 3 can be made, keeping in mind that the total nitrous oxide content of the pregnant uterus is equal to the sum of the nitrous oxide contained in the fetus, the placenta, the uterus itself, and the amniotic fluid.

Having now calculated the nitrous oxide content of the uterus at the end of the period of study we can use this as the numerator in (Equation *1*). In the case used as an example:

$$\text{Uterine Blood Flow} = \frac{N_2O \text{ in uterus}}{(N_2O)\text{ artery} - (N_2O)\text{ uterine vein}} \times 100 = 530 \text{ cc. per minute.}$$

V. Additional data derived from estimated uterine blood flow

Analyses for oxygen and carbon dioxide content are done on the arterial and uterine venous blood samples and on the umbilical arterial and umbilical venous blood. With this knowledge the following calculations can be made:

1. Uterine oxygen consumption = Uterine blood flow $\times [(O_2)$ artery $- (O_2)$ uterine vein$]$;

2. Uterine carbon dioxide production = Uterine blood flow $\times [(CO_2)$ uterine vein $- (CO_2)$ artery$]$;

3. $\text{Uterine RQ} = \dfrac{\text{Uterine carbon dioxide production}}{\text{Uterine oxygen consumption}}$;

4. $\text{Fetal RQ} = \dfrac{(CO_2)\text{ umbilical artery} - (CO_2)\text{ umbilical vein}}{(O_2)\text{ umbilical vein} - (O_2)\text{ umbilical artery}}$;

5. An approximation of the volume of *fetal* blood flowing to the placenta can be reached by assuming that all oxygen used by the uterus is consumed by the fetal tissues. Actually, some oxygen will be consumed by the myometrium so this assumption gives a figure which will err in being too large. It is called "Maximum umbilical blood flow."

$$\text{"Maximum umbilical blood flow"} = \frac{\text{Uterine oxygen consumption}}{(O_2)\text{ umbilical vein} - (O_2)\text{ umbilical artery}} \times 100.$$

[7] In experiments performed on two goats and four sheep the nitrous oxide concentration of umbilical arterial blood was compared with the nitrous oxide concentration of the whole fetus. In four of the six experiments the figures agreed within 0.5 vol. per cent.

TABLE I

Uterine blood flow and related figures

Experiment number	Birth weight of infant Kg.	Uterine blood flow cc./minute	Uterine O_2 consumption cc./minute	Uterine CO_2 production cc./minute	Uterine respiratory quotient	Uterine O_2 consumption / Birth weight of infant cc./minute/kilogram	UBF_E† cc./100 gm./min.
7	3.5	590	17.7	8.3	0.5	5.1	13.4
8	3.1	535	18.2	20.0	1.1	5.9	15.5
10*	3.0	330	16.4	10.8	0.7	5.5	8.8
11*	3.2	560	21.3	23.0	1.1	6.5	13.9
12*	3.1	480	16.4	12.5	0.76	5.3	12.7
14*	3.6	510	31.0	38.0	1.2	8.5	10.9
16	3.7	315	14.8	13.9	0.94	4.0	9.0
18	3.7	840	22.7	27.7	1.2	6.1	22.0
19	2.9	255	13.0	8.7	0.67	4.5	7.8
21	3.2	770	57.0	47.0	0.83	18.0	19.5
24	3.5	700	44.0	40.6	0.92	12.5	13.0
25	2.9	335	21.8	21.5	0.98	7.5	8.0
26	3.3	175		10.9			6.4
20	3.5 & 3.5 Twins	1,150	48.3	47.2	0.98	6.9	18.0
Mean of single pregnancies	3.3	492	24.5	21.8	0.91	7.5	12.4
Standard deviation	.28	195.4	12.7	12.5	0.19	3.8	4.45
Standard error	.078	54.2	3.67	3.47	0.055	1.1	1.23

* Nitrous oxide concentration of umbilical arterial blood assumed to be 3.5 cc. per 100 cc.

† Uterine blood flow calculated upon the assumption of nitrous oxide equilibrium between maternal blood and uterine contents.

RESULTS

The procedure described has been attempted at elective repeat Caesarian section at term in twenty-five women. In ten of these, results are sufficiently complete to permit calculation of uterine blood flow and the supplementary data derived from a knowledge of uterine blood flow. These are summarized in Table I. Four additional experiments (Nos. 10–14) lacked only a determination of umbilical arterial nitrous oxide concentration. These four cases were included by using the average nitrous oxide concentration (3.5 cc. per 100 cc.) found in other experiments in which the same period of nitrous oxide inhalation was employed prior to delivery of the fetus. The variation in this nitrous oxide concentration was small (2.8 to 3.9 cc. N_2O per 100 cc. blood in nine experiments).

One of the subjects studied in whom data for calculation of uterine blood flow is complete had a twin pregnancy. The uterine blood flow and values calculated from it are listed separately in this case.

Uterine blood flow determined upon the assumption of nitrous oxide equilibrium between maternal blood and uterine contents is included in the column marked UBF_E for comparison with figures in the literature (7).

DISCUSSION

From a consideration of Table I, it can be seen that the uterine blood flow calculated by this method and using these assumptions varies widely from patient to patient. Such variation is to be expected in any physiological function studied in a series of individuals. All patients studied were normal multiparae undergoing repeat Caesarian section at term. Spinal anesthesia was used in the cases reported here. The variability in results may be due, in part, to limitations in the experimental technique, particularly in sampling "mixed" venous blood from the pregnant uterus. That samples drawn from any single vein are representative of the average (or "mixed") venous blood is open to question.

On one occasion samples were drawn simultaneously from each of two large veins which drained the right and left uterine parametrial regions respectively. The concentrations of nitrous oxide were identical in the two veins.

We have tried to minimize this possible error by selecting only large veins and by passing the catheter well downstream in them, so that an average of several large tributary blood streams could be expected.

Even with this possible source of error only two of the calculated flows are below 300 cc. per minute, and only three of the single pregnancies studied have a calculated flow above 600 cc. per minute. The average for all single pregnancies studied is approximately 500 cc. per minute. This average figure is probably an accurate one within the conditions of the study.

Assali, Douglass, Baird, Nicholson, and Suyemoto (7) has also attempted to measure uterine blood flow using nitrous oxide. They made the assumption that nitrous oxide equilibrium exists between uterus and blood at the end of thirty minutes of nitrous oxide administration, and have reported an average flow of 15 cc. per 100 gm. per minute in a series of seven women studied during the last month of pregnancy. If the average weight of the pregnant uterus is 5 kg., a blood flow of 500 cc. per minute would give a figure of only 10 cc. per 100 gm. per minute. As discussed above the assumption of nitrous oxide equilibrium would be expected to give erroneously high results.

The average oxygen consumption per minute of the pregnant uterus in our subjects is 24.5 cc. per minute, and carbon dioxide production, similarly calculated, averages 21.8 cc. per minute. Thus the average respiratory quotient in the uterus at term is 0.91.

Comparison of the oxygen consumption per minute as calculated here with previously reported figures is difficult. Assali and his associates calculated an oxygen consumption of 1.9 cc. per 100 gm. per minute which is far in excess of values recorded in the present study. If we assume, as they do, that the weight of the pregnant uterus is 5 kilograms, its oxygen consumption would amount to 95 cc. per minute on the basis of their average figure for oxygen consumption. Cugell, Frank, Gaensler, and Badger (15) have reported an average increase in maternal oxygen consumption at term of 65 cc. per minute in non-basal subjects. Such subjects are comparable to those used in Assali's study. In basal subjects the increase in maternal oxygen consumption at term averaged 38 cc. per minute in the patients reported by Burwell, Strayhorn, Flickinger, Corlette, Bowerman, and Kennedy (16).

The marked difference between the figure for oxygen consumption presented here and that obtained by Assali is due not only to the larger uterine blood flow he reported but also to the large arteriovenous oxygen difference (average 12.7 vol. per cent) across the uterus in his seven cases. The average arteriovenous oxygen difference in our series is 4.8 vol. per cent in the single pregnancies. Barron (4) found an average arteriovenous oxygen difference of 3.8 vol. per cent across the uterus of five sheep near term.

Determinations of the oxygen consumption of the fetus were made by Barcroft, Herkel, Hill, Kennedy, and Mason (1, 2, 17) by a variety of methods. Values ranged from 4 to 8 cc. per kg. per minute in the sheep at term to about 8 cc. per kg. per minute for the rabbit embryo at term. Calculation of oxygen consumption in the human newborn gives figures of between 6 and 8 cc. per Kg. per minute (18), a figure of the same magnitude as that determined for the sheep and rabbit. If we assume that the myometrium weighs 1 kg., that the average placental weight is 550 grams, and that these tissues consume oxygen in the same proportion to weight as does the fetus, the average oxygen consumption per kilogram of tissue is 5.05 cc. per Kg. per minute. This figure appears to be of the proper order of magnitude, when compared with the animal observations mentioned above.

The uterine respiratory quotient is included in the Table for the sake of completeness. At present all that can be said about it is that the average respiratory quotient is similar to that of the whole adult under conditions of the "steady state."

The uterine blood flow calculated for the patient with a twin pregnancy is worthy of comment. Each of the babies weighed 3.5 Kg. at birth, and each had a concentration of nitrous oxide in its umbilical arterial blood at delivery which was comparable to that seen in the single pregnancies studied. Thus it appears that the quantity of maternal blood circulating through the uterus per minute is about twice that of the average single pregnancy. This finding is strengthened by the fact that the arteriovenous oxygen difference across the uterus in this patient is al-

most identical with the average found in the single pregnancies.

SUMMARY

A method for estimating the quantity of maternal blood flowing through the pregnant uterus at term has been developed. Estimation of uterine blood flow permits the calculation of several important features of gestational metabolism.

Results obtained by applying this method to 14 normal women at term Caesarian section are reported. The average uterine blood flow in 13 single pregnancies was estimated at 500 cc. per minute and the average oxygen consumption of the uterus and its contents was calculated to be 25 cc. per minute. In one patient with a twin pregnancy the uterine blood flow and uterine oxygen consumption were approximately twice the magnitude of the average of the single pregnancies.

The calculated uterine oxygen consumption per kg. of tissue is in general conformity with data previously obtained by other methods in lower animals.

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17α-HYDROXYPROGESTERONE AND 21-DESOXYHYDROCORTISONE; THEIR METABOLISM AND POSSIBLE ROLE IN CONGENITAL ADRENAL VIRILISM[1]

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Although virilism due to bilateral adrenal hyperplasia is a relatively rare condition, this syndrome has stimulated much interest and research. Previous work has demonstrated that the adrenals of patients with this disease respond differently from the normal when they are further stimulated with exogenous ACTH. Lewis and Wilkins (1), Bartter and his associates (2), and Jailer, Louchart, and Cahill (3) have shown that although the administration of ACTH to patients with congenital adrenal hyperplasia results in a further increase in the already elevated urinary 17-ketosteroids, other responses are different from those seen in normal subjects. For example, following the administration of ACTH to patients with adrenal virilism there is no appreciable rise in urinary corticoids, measured as reducing or formaldehydogenic steroids, no decrease in the circulating eosinophils, and no sodium retention. Furthermore, Kelley, Ely, and Raile (4), and then subsequently Bayliss, Broadbent, and Steinbeck (5), Bongiovanni, Eberlein, and Cara (6) and Christy, Wallace, and Jailer (7) have shown that there is no increase in plasma corticoids in the great majority of these patients under these circumstances.

That the administration of cortisone or hydrocortisone to patients with congenital virilism results in a fall in the urinary 17-ketosteroids and a regression in the characteristic virilizing symptoms of this disease was first demonstrated by Wilkins, Lewis, Klein, and Rosemberg (8) and confirmed by others (2, 9, 10). It is generally accepted that this effect of cortisone is mediated through the inhibition of ACTH secretion by the pituitary.

The underlying steroidal pathology in the adrenals of these patients has been the subject of some speculation. Bartter, Albright, Forbes, Leaf, Dempsey, and Carroll (2) have postulated that this condition is characterized by an increased secretion of adrenal androgen(s) and a decreased production of hydrocortisone. Consequently, an increased amount of ACTH would be required to stimulate the adrenal to secrete the normal amount of hydrocortisone needed for homeostasis. Excessive amounts of circulating ACTH have been found in the blood of such patients (Sydnor, Kelley, Raile, Ely, and Sayers [11]). Apparently, the excessive amounts of adrenal androgen(s) are incapable of inhibiting ACTH secretion. Jailer (12) suggested that the defect in this syndrome may reside in the enzymatic hydroxylation mechanisms which had previously been shown by Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker, Pincus, Hayano, and Dorfman (13–15) to be essential for the biosynthesis of Compounds B and F from simpler steroidal intermediates. This group had previously demonstrated that surviving adrenal tissue possesses hydroxylases which are capable of introducing hydroxyl groups into certain C-21 steroids at carbons 11, 17 and 21, to yield the adrenal hormones: corticosterone and hydrocortisone (15). The simplest C-21 precursor which can be hydroxylated in this fashion may be pregnenolone, a substance which has been proven to be an intermediate in the formation of these hormones from cholesterol (16, 17).

The possibility that one of the important metabolic defects in congenital adrenal hyperplasia may be the inability of the adrenal gland to accomplish the complete enzymatic hydroxylation of the steroidal precursors has been suggested (12). Such a failure might result in the formation of partially hydroxylated secretory products, some of which could be androgenic in nature or others which could be converted to androgens during catabo-

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TABLE I

Urinary metabolites identified in patients with congenital adrenal hyperplasia

Steroid	Mg./day								
	J. B.	R. K.	D. P.	E. B.	S. B.	L. Y.	R. E.	S. P.	J. L.
	♀	♀	♀	♂	♀	♂	♀	♀	♀
Dehydroisoandrosterone			9.1						7.8
Androsterone	3.8	4.0	4.4	10.8	3.2	3.9	10.8	3.2	12.7
Etiocholanolone	2.1	2.1	5.6	4.2	1.6			0.6	3.9
11 Keto-etiocholanolone	1.7				1.8		1.8		3.9
11β-hydroxyandrosterone	2.3	4.0	6.7	7.3	2.0	1.7			
11β hydroxy-etiocholanolone			trace						
Pregnanolone		3.1				1.8			
11 Keto-pregnanolone	1.6	5.9			2.2	17.0	4.6	2.1	
17α hydroxy-pregnanolone	6.9			5.9		2.9		1.2	

lism. Two such intermediary steroids, 17α-hydroxyprogesterone and 21-desoxyhydrocortisone, have been implicated in this syndrome (12). In this study a comparison of the urinary steroidal metabolites resulting from the exogenous administration of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone with those steroids known to be found in the urine of patients with untreated congenital adrenal hyperplasia was made.

Pfiffner and North (18) have isolated 17α-hydroxyprogesterone from extracts of adrenal tissues and demonstrated that it is androgenic. Gassner (19) has recently reassayed this steroid and found that while it was not active when applied locally to the cock's comb it has about one-half the androgenic activity of methyltestosterone when it is administered orally to cockerels. The administration of 17α-hydroxyprogesterone to patients (20) and to rats (21) results in an increase in the urinary excretion of neutral 17-ketosteroids. It has been shown by Fukushima, Dobriner, and Gallagher (22), by means of deuterium-labeled 17α-hydroxyprogesterone, that this adrenal product is metabolized to androsterone and etiocholanolone. Bongiovanni, Eberlein, and Cara (23) have demonstrated that 17α-hydroxyprogesterone is also metabolized to pregnane-3α, 17α, 20α-triol, a steroid characteristically found in the urine of patients with adrenal virilism (6, 24).

21-Desoxyhydrocortisone, another possible partially hydroxylated intermediate of adrenal hormone biosynthesis, has not as yet been associated with any biological activity, nor has its metabolism been previously investigated. The urinary products resulting from the administration of the closely related 21-desoxycortisone have been studied by Burstein, Savard, and Dorfman (25) who have administered it to two patients with rheumatoid arthritis and have recovered from the urine 11-keto-etiocholanolone, 11β hydroxy-etiocholanolone, 11β-hydroxyandrosterone and 21-desoxy-tetra-hydrocortisone.

The objective in this investigation was to determine whether the urinary products resulting from the administration of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone are similar to and could account for the known urinary metabolites found in adrenal virilism. For comparison, the endogenously excreted steroids in the urine of nine untreated patients with congenital adrenal hyperplasia were determined by essentially identical methods (Table I). In addition to the compounds identified in Table I, others have reported the isolation of pregnane 3α, 20α, diol; pregnane-3α, 17α, 20α-triol (26) and pregnanetriol-11-one (reviewed in 27), compounds which were not detected by the techniques employed in this study.

METHODS AND MATERIALS

Patients who were chosen for the study of the metabolism of exogenously administered steroids excreted but minimal amounts of endogenous 17-ketosteroids.

17α-Hydroxyprogesterone [2] was administered orally to eight patients as a water suspension or in gelatine capsules. Each patient received a total of between 600 and 1,000 mg. in divided doses over a two or three-day period. The patients used were as follows:

1. A 40-year-old female Addisonian patient maintained on 25 mg. a day of oral cortisone (F. S.).
2. A 55-year-old man with hypopituitarism (S. W.).

[2] The 17α-hydroxyprogesterone was kindly supplied by the Syntex Corporation and the 21-desoxyhydrocortisone by the Upjohn Company.

3. A 21-year-old male Addisonian patient whose only replacement therapy consisted of implanted pellets of DCA (J. R.).

4. A 55-year-old man with hypopituitarism who received 15 mg. a day of thyroid extract (G. S.).

5. An 11-year-old female pseudohermaphrodite whose urinary 17-ketosteroids were being suppressed by means of daily doses of 50 mg. of parenteral cortisone (F. K.).

6. A 17-year-old female pseudohermaphrodite whose therapeutic regimen consisted of 100 mg. of parenteral cortisone administered thrice weekly (J. L.).

7. A 10-year-old female pseudohermaphrodite who received 100 mg. of cortisone parenterally thrice weekly in order to maintain her urinary 17-ketosteroids close to normal (R. E.).

8. A 6-year-old female pseudohermaphrodite who was receiving 100 mg. per day of parenteral cortisone to suppress her 17-ketosteroids (S. P.).

21-Desoxyhydrocortisone was administered to two patients, both of whom received 300 mg. daily for three days by the oral route. L. B. was a 49-year-old male with hypoadrenalism and hypothyroidism, and H. H. was a 41-year-old housewife who had undergone bilateral adrenalectomy previously because of Cushing's syndrome due to bilateral adrenal hyperplasia. She required 25 mg. per day of oral cortisone for maintenance. The latter patient also received 17α-hydroxyprogesterone several days prior to the administration of 21-desoxyhydrocortisone.

The total neutral urinary 17-ketosteroids were determined by the method of Holtorff and Koch (28) after acid hydrolysis on aliquots of complete 24-hour urine specimens. Another aliquot of urine was hydrolyzed with glucuronidase and the method of Sommerville, Marrian, and Kellar (29) employed to estimate the amounts of pregnanediol. This method probably determines pregnanetriol as well (23).

Still another portion (approximately 1 liter) of some of the 24-hour urines was processed in the following manner: The urine was acidified to 2N with 50 per cent sulfuric acid and then extracted with ether for 24 hours in a continuous extractor. The ether extract was washed with saturated Na_2CO_3, 10 per cent NaOH and water until neutral and then evaporated to dryness. The above alkaline washings were added to the residual urine and the mixture treated with ten volumes per cent of hydrochloric acid. The mixture was then boiled for 20 minutes and extracted continuously a second time with ether for 24 hours. The ether extract was treated as above and combined with the first ether extract. The dried combined extract was dissolved in 10 ml. benzene and chromatographed on an alumina column. In the first several chromatograms elution was carried out by the Dingemanse method (30) and later by the gradient elution method of Lakshmanan and Lieberman (31). Zimmermann reactions were performed on 1 ml. aliquots of the eluates and the results plotted (mcg. per ml.) against the fraction number. The 17-ketosteroids resolved by the chromatographic separation were identified by means of infrared spectrometry.

RESULTS

Congenital adrenal hyperplasia

The amounts of the urinary metabolites found in a 24-hour urine specimen from each of nine untreated patients with congenital adrenal hyperplasia, ranging in age between 5 and 17 years are shown in Table I.

17α-Hydroxyprogesterone

The extract from at least one control urine from each patient who subsequently received this substance was resolved by chromatographic analysis. In only two cases were any ketosteroids identifiable. In J. L., 0.7 mg. 11β-hydroxyandrosterone was found and in S. P., 1.8 mg. 11β-hydroxyandrosterone, 0.8 mg. 11-keto-etiocholanolone and 1.3 mg. 17α-hydroxy-pregnanolone were found.

TABLE II

Effect of 17α-hydroxyprogesterone on the urinary excretion of neutral 17KS and "pregnanediol"

	1 F. S.		2 S. W.		3 J. R.		4 G. S.		5 F. K.		6 J. L.		7 R. E.		8 S. P.	
Day	17KS	P.†	17KS	P.	17KS	P.	17KS	P.	17KS	P.	17KS	P.	17KS	P.	17KS	P.
1	1.1	0.2					0.7									
2	1.2		1.2		2.7	1.6	0.4		0.6				2.5		5.	
3	2.2		0.9	3.2	4.3		0.0	0.6	4.2	1.4	6.9	0.1	4.2	2.3	7.6	4.3
4	3.5*		4.3*		9.6*	8.0	6.5*		4.6*		29.6*		11.8*		—*	
5	8.6*	9.9	8.3*	8.0	13.5*		10.5*		13.5*	0.6	39.5*	7.2	19.2*	4.5	24.4*	19.4
6	12.4*		6.5*		5.2		19.5*	4.7					24.5*		7.7	
7			3.4				2.3						21.5		6.1	
8	(750)‡		(600)		(1,000)		(900)		(900)		(900)		(900)		(900)	

* Day 17α-hydroxyprogesterone was administered.
† P. = "Pregnanediol" as measured by the Sommerville-Marrian method.
‡ Figure in parentheses—total mg. administered.

TABLE III

The effect of orally administered 17α-hydroxyprogesterone and 21-desoxyhydrocortisone on urinary 17KS

	H. H.		L. B.	
Day	17KS	Treatment	17KS	Treatment
1	4.4		4.0	
2	6.9		5.0	
3	17.9	300 mg. 17-OH-prog./day for 3 days	5.9	
4	45.8		7.0	
5	55.6		19.4	300 mg. 21-Desoxy F/day for 3 days
6	22.1		32.2	
7	19.5		20.8	
8	30.6	300 mg. 21-Desoxy F/day for 3 days	9.3	
9	26.6		5.7	
10	25.2		3.8	
11	10.2			
12	8.2			

The oral administration of 17α-hydroxyprogesterone resulted in an increase in the total neutral urinary 17-ketosteroids in all patients (Tables II and III).

Urinary extracts obtained from each patient during the treatment period were also analyzed. Androsterone and etiocholanolone were invariably found. The percentage yields were not easily determined since only one day's urine was analyzed rather than a pool of all the urines excreted during the treatment period. Nevertheless, the data permit the observation of an approximate ratio of about 1 : 5 of androsterone to etiocholanolone (5α–5β). In contrast to the above, the urines collected for the three days while the patient, H. H., was receiving 17α-hydroxyprogesterone were pooled and analyzed (Table III). Five and eight-tenths mg. of androsterone and 26.1 mg. etiocholanolone were found during this period, whereas no urinary metabolites could be identified during the control period.

The administration of 17α-hydroxyprogesterone also resulted in an increased excretion of the "pregnanediol-pregnanetriol complex" as measured by the Sommerville, Marrian, and Kellar method (29), Table II. The presence of both pregnanediol and pregnanetriol in this fraction was suggested when the chromatographic method of Bongiovanni and Clayton (32) was employed and the sulfuric acid chromogenic absorption curves were compared to those of standard compounds. In one patient (S. W.) for example, the pregnanediol rose from 1.8 mg. to 19.4 mg. and the pregnanetriol value from 1.8 to 15.0 mg. after the administration of 17α-hydroxyprogesterone.

In addition, 3α-hydroxy-pregnan-20-one was found in the urine of one patient and 17α-hydroxypregnanolone in the urine of four patients who had received 17α-hydroxyprogesterone. The latter metabolite was identified by means of infrared spectrometry and the former by means of infrared spectrometry, melting point and mixed melting point.

21-Desoxyhydrocortisone

Two patients received 300 mg. per day for three days of 21-desoxyhydrocortisone by mouth. This regimen resulted in an appreciable rise in the urinary excretion of the total neutral 17-ketosteroids (Table III).

In spite of the Zimmermann chromogen found during the control period in the urines of H. H., and L. B., no identifiable ketosteroids were found in the urine of H. H., whereas but a fraction of a milligram of etiocholanolone was detected in the control urine of L. B.

TABLE IV

The urinary metabolites isolated after the administration of 21-desoxyhydrocortisone

L. B.	mg.	H. H.	mg.
11β-hydroxyandrosterone	20.5	11β-hydroxyandrosterone	9.5
11-keto-androsterone	20.7	11 keto-etiocholanolone	11.5
Etiocholanolone	4.3	11 keto-pregnanolone	11.6
11β-hydroxy-etiocholanolone	12.2		
11-keto-etiocholanolone	16.3		
11-keto-pregnanolone	7.8		
21-desoxy-tetrahydrocortisone*			
21-desoxy-tetrahydro-hydrocortisone†			

* 3α, 17α dihydroxy-pregnane 11,20 dione.
† 3α, 11β, 17α trihydroxy-pregnan-20-one.

The urinary metabolites found as a result of the administration of the test substance are shown in Table IV. 11-Keto-pregnanolone was identified by melting point and mixed melting point as well as infrared spectrometry. Aliquots of urine from the control period and during the time patient L. B. was receiving 21-desoxyhydrocortisone were analyzed by the method of Zondek and Finkelstein (33) for neutral fluorogenic steroids. The substance responsible for this fluorescence was subsequently shown by Finkelstein, von Euw, and Reichstein (27) to be pregnanetriol-11-one. During the control period no fluorogenic substance could be detected, whereas the equivalent of 6 mg. was estimated in the pooled urines during the treatment period.

Another new metabolite of 21-desoxyhydrocortisone was identified in the urine of both patients and was shown to be 3α, 11β, 17α-trihydroxy-pregnan-20-one, (isolated as its Δ^9 analogue) (34).

DISCUSSION

The data presented above indicate that 17α-hydroxyprogesterone is metabolized to the 17-ketosteroids, etiocholanolone and androsterone.

3α-Hydroxy-pregnan-20-one and 17α-hydroxy-pregnanolone have also been identified as metabolites of 17α-hydroxyprogesterone, whereas the presence of pregnanetriol and pregnanediol has been indicated by means of their absorption spectra in sulfuric acid. The administration of 21-desoxyhydrocortisone, on the other hand, results in the appearance in the urine of the following 17-ketosteroids: 11β-hydroxyandrosterone, 11-keto-androsterone, 11-keto-etiocholanolone, 11β-hydroxy-etiocholanolone. In addition, 11-keto-pregnanolone, 21-desoxy-tetrahydrocortisone and 21-desoxy-tetrahydro-hydrocortisone were found. The possible presence of pregnanetriol-11-one was suggested by the fluorescent technique of Zondek and Finkelstein (33). Figures 1 and 2 illustrate these steroidal conversions.

The finding of pregnanolone-3α and 11-keto-pregnanolone as metabolites of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone respectively demonstrates the body's ability to dehydroxylate at the C-17 position. The implication of these results will be more fully discussed in a subsequent publication (35).

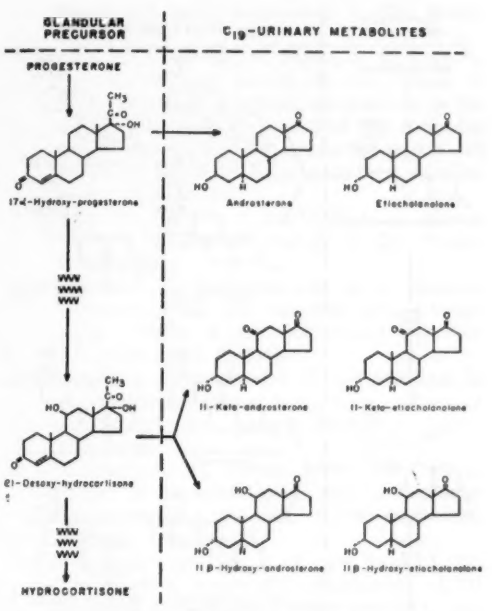

FIG. 1. THE URINARY C-19 METABOLITES OF 17α-HYDROXYPROGESTERONE AND 21-DESOXYHYDROCORTISONE

Since the administration of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone produces a urinary steroidal pattern somewhat similar to that found in patients with congenital adrenal hyperplasia (compare Tables I with Figures 1 and 2) the possibility exists that these glandular products play an important role in this disease. This becomes all the more possible since 17α-hydroxyprogesterone has been shown to serve as a direct precursor of hydrocortisone in various biosynthetic experiments. While this has not been reported for 21-desoxyhydrocortisone, Meyer, Rodgers, and Pincus (36) have demonstrated that 21-desoxycortisone may be hydroxylated to produce cortisone *in vitro*.

As has been noted earlier, patients with congenital adrenal hyperplasia respond differently from the normal when exogenous ACTH is administered in that they apparently do not produce additional amounts of hydrocortisone. The increases in urinary ketosteroids and in the pregnanediol-pregnanetriol complex that do follow the administration of the trophic hormone suggest that the adrenals of these patients are capable of

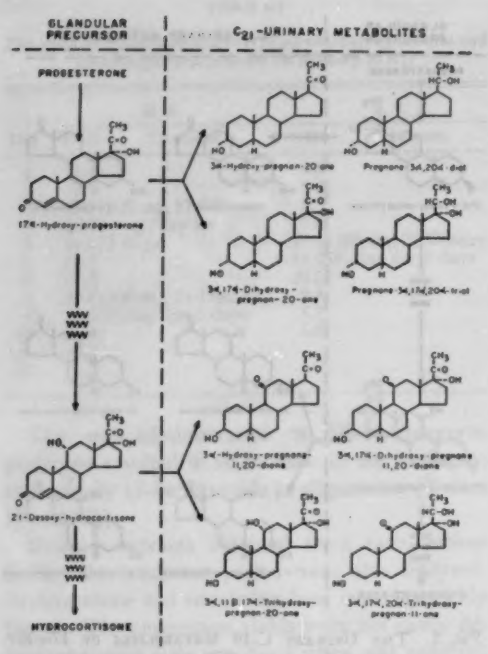

FIG. 2. THE URINARY C-21 METABOLITES OF 17α-HYDROXYPROGESTERONE AND 21-DESOXYHYDROCORTISONE

synthesizing adequately some of the precursors of hydrocortisone. The fact that the urinary metabolites found in congenital adrenal hyperplasia are structurally related to and in many cases identical with those found after the exogenous administration of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone indicates that the inability of such adrenals to synthesize hydrocortisone may be due to some defect in the enzymatic hydroxylation mechanisms which are necessary for the complete biosynthesis of hydrocortisone. A specific enzymatic defect in either the 11β-hydroxylase or the 21-hydroxylase systems has been implicated in adrenal hyperplasia (12, 37). However, the patterns of urinary steroid excretion given in Table I do not seem to indicate a single cause for this syndrome but rather they point to several individual defects, any of which could result in a failure to biosynthesize hydrocortisone in sufficient amounts. The absence of excessive amounts of 11-oxygenated C-19 metabolites in some patients (J. B., R. E., S. P., and J. L.) would suggest that the 11β-hydroxylation mechanisms in these patients is the main deficiency. In those patients (R. K., D. P., and E. B.) wherein large amounts of these metabolites were isolated, the principal block may be in the C-21 hydroxylation mechanism. Likewise, the isolation of large amounts of 11-keto-pregnanolone (L. Y.) also points to a failure of C-21 hydroxylation and possibly in addition to an inability to hydroxylate at C-17. Relative failure of this latter enzymatic process may also account for the isolation of excessive amounts of pregnanolone (R. K. and L. Y.). Bongiovanni and Eberlein (38) have presented evidence which indicates that in some children with adrenogenital syndrome hydroxylation at C-21 is not impaired.

The evidence accumulated to date does not appear to indicate that congenital adrenal hyperplasia is characterized by a single enzymatic block. Rather it seems likely that the similarity in all cases is a defect in hydroxylation; in some cases this occurs with those enzymes which hydroxylate at C-11, in others with those systems responsible for hydroxylation at C-21, or still others with the C-17 hydroxylating mechanism. There may be instances where the 3β-hydroxylase (3β-hydroxy $-\Delta^4$-3-ketone) may be impaired and this could account for the large amounts of dehydroisoandrosterone occasionally found (D. P. and J. L.). In general, any event which results in diminished production of the end product, Compound F, leads to an accumulation of partially hydroxylated intermediates which upon escaping from the adrenal gland are metabolized along well-known paths. Some of the intermediates or their metabolites may be androgenic and could therefore be reponsible for the virilism observed. It should be pointed out that the virilism of patients with congenital adrenal virilism differs somewhat from that observed in patients with virilizing adrenal tumors or patients receiving large amounts of testosterone. Although the former patients do exhibit axillary and pubic hair together with an enlarged phallus or clitoris, body hirsutism is not usually encountered. The women may even have a female pubic escutcheon and they rarely have to shave. This is in contrast to the latter patients who exhibit marked body hirsutism.

In addition to enzymatic failures, Dorfman (39) has proposed that the virilism of the adrenogenital syndrome is due to the synthesis of large

amounts of C-19 androgens, *e.g.*, Δ^4-androstene-3,17-dione, *etc.*, by the adrenals and that these arise from acetate or cholesterol but not via C-21 precursors, as, for example, pregnenolone. According to this hypothesis the synthesis of these adrenal androgens are interrelated with the adrenal secretion of the C-21 steroids since both processes are stimulated by the excessive amounts of ACTH thought to be present in the blood of such patients. It is difficult to explain by this concept the normal urinary 17-ketosteroid values often observed in patients with Cushing's syndrome also due to bilateral adrenal hyperplasia (40). Moreover, as Bongiovanni and Clayton (32) have pointed out, the excretion of abnormal amounts of the C-21 steroid, pregnane-3α, 17α, 20α-triol is more characteristic of adrenal hyperplasia than is an elevated 17-ketosteroid level.

The virilism due to adrenal tumors, on the other hand, may be considerably different in nature since the principal urinary steroid found in these instances is dehydroisoandrosterone whose metabolic precursors are still uncertain (41).

SUMMARY

An attempt to reproduce the urinary steroidal pattern characteristic of congenital adrenal virilism by means of the administration of 17α-hydroxyprogesterone and 21-desoxyhydrocortisone has been made. The urinary metabolites of these steroids have been identified as: etiocholanolone; androsterone; 11-keto-etiocholanolone; 11-hydroxy-etiocholanolone; 11β hydroxyandrosterone; pregnanolone; 17-hydroxy-pregnanolone and 11-keto-pregnanolone. The presence of pregnanediol, pregnanetriol and pregnanetriol-11-one have been indicated by means of chromogenic reactions.

The following hypothesis is presented: Congenital adrenal hyperplasia is accompanied by enzymatic defects in the hydroxylation mechanisms, which results in the piling up of partially hydroxylated hydrocortisone precursors. These escape into the circulation and are metabolized along well-known pathways resulting in the production of androgens.

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PROTEIN INTERACTIONS OF GENTISIC ACID AND CERTAIN OF ITS OXIDATION PRODUCTS [1]

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Although salicylate was introduced eighty years ago in the treatment of rheumatic fever (1), the mechanism of its effect is still unknown. Speculation concerning the mode of action of salicylate and its congeners has run from considerations of one or another of the physico-chemical properties of phenolic acids to a supposed effect on the pituitary-adrenal axis (2–4). In a recent study of tissue interactions of certain salicylic acid derivatives, Helander attempted to demonstrate selective localization of sodium salicylate in connective tissue using fluorescence microscopy but was unable to do so (5). From his investigations he concluded that attachment of both a carboxyl- and an amino- group to the aromatic ring is necessary for connective tissue binding. These investigations did not include gentisic acid, a diphenol which takes on special importance because in the human subject as well as in animals an appreciable portion of administered salicylate (5 to 8 per cent) is oxidized and excreted as gentisate (6–8).

Numerous workers (9–12) have now confirmed the observation of Meyer and Ragan in 1948 (8) that gentisate has an antirheumatic effect entirely comparable to that of salicylate itself. The present report concerns the affinity of gentisic acid and its oxidation products for connective tissue, an experimental approach to the hypothesis that oxidation of the antirheumatic phenols is essential to their action.

METHODS

Observations on distribution of gentisic acid using its fluorescence in ultra-violet light. Sodium gentisate was prepared in distilled water in concentrations of 0.5 to 5.0 per cent. Mice were injected with 1.0 ml. intravenously and sacrificed at intervals. Dissection and observation were carried out in the near ultra-violet light (Hanovia Inspectolite, wave-length maximum 3,660 Å).

Equilibrium dialysis. The method used by Taggart (13) was employed, with the following modifications: Dialysis was carried out at 0° C., and tubes were inverted frequently rather than shaken constantly. Duplicate determinations were made, gentisic acid being added in one instance to the inside of the cellophane bag and in the second to the buffer on the outside. Equilibrium was attained in 24 hours. Buffer was 0.067 M sodium phosphate, pH 7.0 and 7.5, in 0.05 M NaCl, except when procollagen was tested. In this case acetate buffer pH 4.0 was used. Gentisic acid was determined by the method of Roseman and Dorfman (14).

Tissue Extracts. Procollagen was prepared from rat tails by the method of Oriekwovitch (15). Beef tendomucoid was extracted according to the method of Bergmann and Stein (16). "Chondromucoid" from beef was prepared according to the method given in Hawk, Oser, and Summerson (17). Gelatin-chondroitin sulfate was generously provided by Dr. Karl Meyer of this Department.

Oxidation in air of gentisate. A 7.5 per cent solution of sodium gentisate in phosphate buffer 0.05 M pH 7.5 in Erlenmeyer flasks plugged with cotton was shaken at 37° C. for 18 to 24 hours. No bacterial growth was found.

Oxidation in air of gentisate with added tissues and particles. Solutions of sodium gentisate in phosphate buffer 0.05 M, pH 7.5, were prepared in concentrations ranging from 20 to 0.04 mg. per cent. To 15 ml. of the solutions were added in separate experiments pieces of 1) dried collagen, about 150 mg.; 2) hide powder, about 100 to 125 mg.; 3) cartilage powder, about 450 mg.; 4) non-nutritive cellulose; 5) sand; 6) filter paper particles; 7) mouse hair; 8) epilated mouse skin 0.5 cm. × 0.5 cm. These flasks were shaken for 3 hours at 37° C.

Collagen was prepared by the method of Einbinder and Schubert (18). Cartilage powder and hide powder were commercially available. The non-nutritive cellulose used was that of the Nutritive Biochemical Corp.; the filter paper No. 1 Whatman cut into 0.5 × 0.5 cm. squares.

Serum fractionation. Globulin was precipitated from human serum by the addition of Na_2SO_4 22 per cent w/v. Globulin and albumin were dialyzed continuously against phosphate buffer until no further SO_4^{--} could be detected in the dialysate.

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Exhaustive dialysis of oxidation products of gentisic acid. A 7.5 per cent solution of sodium gentisate in phosphate buffer pH 7.5, 0.05 M, was shaken at 37° C. until no further increase in brown color occurred, as indicated by constant optical density. To 4.0 ml. of this solution was added 1) albumin content of 1.0 ml. of serum; 2) globulin obtained from 1.0 ml. of serum; 3) phosphate buffer. To 4.0 ml. of the oxidized gentisate was added 1) 1.0 ml. of serum; 2) 3.0 ml. of 7.0 per cent gelatin solution. All were then brought to 7.0 ml. with phosphate buffer. Five ml. of each of these combinations were placed in cellophane bags and dialyzed in the cold against frequent changes of phosphate buffer pH 7.5, 0.05 M, until all gentisic acid had been removed from the bag as shown by lack of fluorescence of the dialysate.

Paper electrophoresis. Separations were carried out using phosphate buffer pH 7.5, $\Gamma/2 = 0.1$, on Whatman paper No. 3 in a lucite chamber, 12 to 15 volts per cm. paper, for a run of 3 to 4 hours. Papers were examined in daylight and in the ultra-violet.

RESULTS

The in vivo affinity of gentisate for connective tissues

The distribution of gentisic acid following oral or intravenous administration has not been studied previously. In ultra-violet light, gentisic acid displays a striking bluish-white fluorescence so intense that detection of trace amounts is possible (19). After intravenous injection into mice fluorescence of the connective tissues could readily be appreciated and was greatest in skin, tendons, bones, and cartilages (Figures 1 and 2).

FIG. 1. FLUORESCENCE OF SUBCUTANEOUS TISSUES 30 MINUTES AFTER INTRAVENOUS GENTISATE—CONTROL MOUSE ON THE RIGHT

Gentisic acid is excreted rapidly in the urine. In keeping with this, the fluorescence of the skin and the tendons largely disappeared within two hours. However, cartilages and bones retained the whitish fluorescence for 5 to 6 hours and sometimes longer, perhaps largely because these structures have a meager blood supply, although the possibility may be allowed that a somewhat firmer attachment of the drug could also contribute to its persistence in these tissues. In any case, if the distribution of fluorescence may be taken to reflect binding of gentisate by connective tissues, the interaction appears to be easily reversible.

Binding of gentisate in vitro

A first approximation to the study of the *in vitro* binding of gentisate by tissues was made by exposing slivers of pigs' knuckles and pieces of cartilage, muscle, liver, kidney, and tendon from freshly sacrificed mice to 1 per cent solutions of sodium gentisate for 30 minutes and then washing them briefly in running tap water. Fluorescence could now be demonstrated in each fragment. Immersion in saline for several hours resulted in disappearance of fluorescence from all tissues except cartilage and tendon.

With equilibrium dialysis no binding of gentisate could be demonstrated for any of the following tissue extracts: beef tendomucoid, beef chondromucoid, rat procollagen, 1 per cent gelatin, 1 per cent gelatin plus heparin, 1 per cent gelatin plus glycogen. Suspensions of an insoluble gelatin-chondroitin sulfate complex did not bind gentisate, nor did suspensions of hide powder, or collagen powder.

However, considerable binding of gentisic acid by both human and bovine serum albumin was found in molar ratios corresponding to those that mark the interaction of many pharmacologic agents, *e.g.*, salicylate, with serum albumin (20). At concentrations of free gentisate 10^{-3} molar, using 65,000 as molecular weight of albumin, about 3 moles of gentisate were bound per mole of albumin. The amount of serum albumin present accounted for all the binding by serum. All of the gentisic acid could be removed by exhaustive dialysis.

Serum albumin was thus the only protein, soluble or insoluble, to bind gentisic acid *in vitro* in measurable amounts.

FIG. 2. RAT THORACIC CAGE TO SHOW FLUORESCENCE OF BONE AND CARTILAGE AFTER INTRAVENOUS GENTISATE—CONTROL ON RIGHT

Binding of oxidation products of gentisic acid by proteins

Gentisic acid is the reductant in an oxidation-reduction system [E_0[3] 0.796 m.v. at pH 0.028 (21)] of which the oxidant, gentisoquinone, is unstable and has not been isolated. Gentisate is readily oxidized in air at neutral pH to brown or reddish brown pigments, presumably quinone which has in some part polymerized. The reaction appears to reach an end point after about 24 hours of vigorous shaking at 37° C. Since the oxidation products have not been identified and suitable methods for their measurement do not exist, it is not possible to provide accurate data on the amounts of gentisate that may be oxidized. Under the experimental conditions and at neutral pH, most of the gentisate (± 90 per cent) probably remained in the reduced form. The presence of pigmented oxidation products can be easily appreciated by the eye in dilute solutions of gentisate in the range of 10^{-6} to 10^{-5} molar.

When pieces of dried collagen or hide powder were shaken with solutions of gentisate at pH 7.5, definite pink to reddish brown coloration of the proteins could be noted within three hours. This pigmentation was detectable at concentrations of gentisate as low as 0.08 mg. per cent (5×10^{-6} M), and was not removed by washing in running tap water for 24 hours or by treatment with organic solvents. Controls containing non-protein particles failed to show pigmentation.

Application of paper electrophoresis to the separation of oxidation products of gentisate

Solutions of gentisic acid were subjected to electrophoresis on paper, both before and after oxidation in air. The findings were interpreted in terms of the appearance in visible and in ultraviolet light and after spraying with phenol reagents.

Fresh solutions of gentisate appeared to be electrophoretically homogeneous, showing a rapidly moving spot giving whitish fluorescence. After oxidation a more complex but remarkably characteristic and constant pattern could be seen, marked by five separate components. In the ultra-violet there were four discrete spots, *viz.*, (a) a bright yellow spot which remained at the base line; (b) that of gentisate itself; (c) a small orange spot ahead of (b); and (d) a yellow spot which moved fastest of all (Figure 3). All of these areas could be stained with ammoniacal silver nitrate. In daylight there could be appreciated only (e) a single brown spot which overlay the front of the gentisate area.

Electrophoresis was carried out on control solutions of gentisate which had been both oxidized in air and exhaustively dialyzed. Only the brown spot (e above) was detectable, *i.e.*, only one of the components seemed to be non-dialyzable. It had

[3] E_0 = electrode potential at 50 per cent reduction.

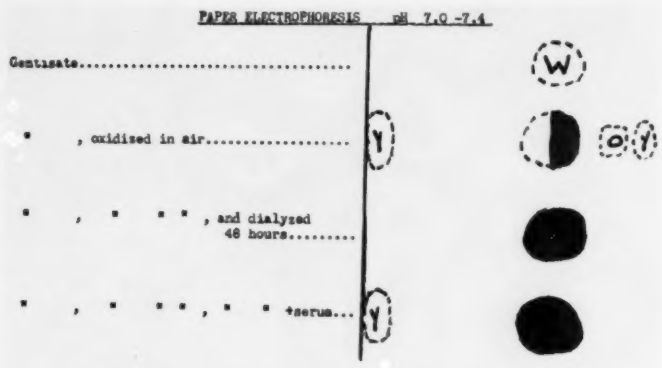

FIG. 3. SCHEMATIC DIAGRAM OF ELECTROPHORETIC PATTERN OF COMPONENTS OF OXIDATION PRODUCTS OF GENTISATE—SEE TEXT

appeared from the earlier experiments noted above that pigmented oxidation products were bound to proteins. Now it became of interest to see if any other of the electrophoretically separable materials might also interact with protein in a similar fashion.

Accordingly, albumin, globulin, and gelatin were added to solutions of gentisate and submitted to exhaustive dialysis. Subsequent electrophoresis showed that one of the fluorescent materials, represented by spot (a), was rendered non-dialyzable in the presence of any of these proteins, *i.e.*, was presumably firmly bound.

DISCUSSION

Gentisate appears to be bound to some degree by living connective tissues, perhaps especially by cartilage and bone. No binding could be shown for fibrous proteins rendered soluble by various means, any of which may have been extreme enough to destroy the chemical affinities possessed by living tissue. It is, of course, possible that the apparent binding to the tissues *in vivo* was due to the presence of extravascular plasma albumin, estimated to make up as much as 50 per cent of the total albumin in the body (22). On this view, failure of solid protein preparations such as collagen powder to bind gentisate could be attributed to loss of albumin in the processing.

On the other hand, any appraisal of the chemical activity of gentisate must consider the ease with which it is oxidized and the reactivity of the oxidation products. It has been shown that oxidation products of gentisate can be separated electrophoretically into four distinct components and at least two of these appear to form complexes with proteins that are not readily dissociated *in vitro*.

It might be objected that these oxidation products are unlikely to form *in vivo* because of the poising action of the tissue elements. In this respect it is of interest to compare the oxidation-reduction system of gentisic acid to that of homogentisic acid; the electrode potentials are comparable (21, 23). In the ochronosis of alkaptonuria (24, 25) the blackness of the cartilages, sclerae, and tendons is ascribed to oxidation products of homogentisic acid (23). In his original description of this disorder Virchow (25) remarked on the curious distribution of the ochre pigment, *i.e.*, its predilection for the connective tissues, and pointed out that most prominent among these were cartilage and bone which have almost no blood supply. Fishberg and Dolin (23) comment that "these are the regions where there can be no poising effect of hemoglobin. Hence the quinone from the homogentisic acid can be formed." These views could be applied with equal force to gentisic acid. It should also be pointed out that in alkaptonuria the blood concentrations of homogentisic acid may be no higher than 1 to 3 mg. per cent (26), while after oral administration of gentisic acid serum levels as high as 15 to 30 mg. per cent may be obtained (27).

It is noteworthy that quinones of gentisic acid inhibit hyaluronidase (28–30). This fact provides another suggestion that gentisic acid and its oxidation products may influence the metabolism of connective tissues. While direct evidence for the idea that oxidation of the phenols, perhaps to quinones, is important for their anti-inflammatory effect will be difficult to obtain yet the findings so far are consistent with this view, and it does appear that the affinity for connective tissue of gentisate and its oxidation products exceeds that of salicylate itself.

SUMMARY

1. After intravenous injection gentisate is excreted rapidly and soon disappears from most organs except the skeletal connective tissues, in which it remains for a considerable time.

2. *In vitro* binding of gentisate by various proteins demonstrated an attachment to serum albumin in the molar ratios that characterize other cations of similar molecular size.

3. Oxidation products of gentisate can be separated electrophoretically into at least four distinct components. Some of these appear to be firmly bound not only by serum albumin but also by insoluble connective tissue proteins, *e.g.*, collagen.

4. It is suggested that the mode of action of the antirheumatic phenols may involve their oxidation *in vivo* to quinones which interact with connective tissue, *i.e.*, that there is an analogy to the metabolism of homogentisic acid in the alkaptonuric.

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THE RELATION OF NITROGEN RETENTION TO NITROGEN INTAKE IN ADULTS WITH POST-TRAUMATIC MALNUTRITION

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Over a wide range of nitrogen intake, protein-depleted (1, 2) or growing rats (3, 4) retain increasing amounts of nitrogen as the dietary nitrogen is raised provided adequate calories are fed. Malnourished (5) or normal children (6) also increase nitrogen retention as nitrogen intake is raised, although some exceptions have been noted (6, 7). In malnourished adults it is apparent that some similar relationship between nitrogen intake and nitrogen retention is also present. For several reasons, however, the relationship between nitrogen intake and nitrogen retention in malnourished adults has not been well defined. In most adult studies, for example, caloric intake has frequently been substantially altered along with variations in nitrogen intake (8–13). In view of the known effect of non-protein calories on nitrogen metabolism (14) such studies become quite difficult to interpret. Equally difficult to interpret are studies (7, 11, 15) in which fecal nitrogen analysis has been omitted. And finally it should be noted that in many instances study periods have been too short with no account taken of the variable time required for re-equilibration of nitrogen metabolism after a change in dietary nitrogen (9, 10, 16–21).

In the present study, nitrogen retention was measured at various levels of nitrogen intake in a series of patients suffering from malnutrition after severe injury. In most of the patients at least two levels of nitrogen intake were employed, while caloric intake was kept constant. Balance periods were sufficiently long to allow for re-equilibration of nitrogen metabolism after changes in dietary nitrogen. In all studies fecal nitrogen analyses were performed.

MATERIAL AND METHODS

The thirteen subjects of this study were soldiers who had been severely injured or wounded in combat. Their individual case histories are recorded in the appendix. A patient's average body weight before injury was taken to be the pre-injury weight. In patients who had limbs amputated, the estimated weight of the amputated limb was subtracted from the pre-injury weight to give the corrected pre-injury weight. Lean body weight before injury was calculated using Behnke's (22) formula for Navy men; lean body weight in kilograms = 53.9 (surface area in M^2) − 32.6. Lean body weight at the start of study was determined by measurement of skinfold thickness using calipers with the specifications described by Brožek and Keys (23). The formula of Rathbun and Pace (24) was used to convert body specific gravity to per cent body fat. The estimated changes in body composition of each subject are presented in Table I.

All patients except patient number 7 received a liquid diet throughout these studies. Ten patients were studied at two or more levels of protein intake. As the protein intake was varied, the caloric intake and the ratio of carbohydrate to fat were maintained constant by calculation using standard food tables (25). In patients 3

TABLE I

Estimated changes in body composition at start of study

Patient	Percentage losses		
	Body weight	Lean body weight	Body fat
1	14		
2	26	26	33
3	16	13	70
4	39	35	77
5	26	24	72
6	23	19	75
7	32	29	68
8	22	19	57
9	23	21	49
10	23	21	66
11	19		
12	36	35	72
13	26	25	64

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TABLE II

Composition of a representative liquid diet with three levels of protein intake

Food	100 Grams protein			180 Grams protein			270 Grams protein		
	Weight *grams*	Nitrogen *grams*	Energy *calories*	Weight *grams*	Nitrogen *grams*	Energy *calories*	Weight *grams*	Nitrogen *grams*	Energy *calories*
Skim milk powder	140	8.0	507	400	22.8	1,448	700	39.9	2,534
Cream	950	4.4	1,938	560	2.6	1,142	100	0.5	204
Eggs	200	4.1	324	200	4.1	324	170	3.5	275
Sugar	140		539	100		385	75		289
Total		16.5	3,308		29.5	3,299		43.9	3,302

and 5, nitrogen retention was also measured at a second caloric intake. The liquid diets were made up from skim milk powder, cream, eggs and sugar. The daily volume was approximately 1,400 cc. and was divided into six feedings: one glass every two hours from 8 A.M. to 6 P.M. Diets were prepared a day in advance; ingredients were weighed, and mixed with a measured volume of water in an electric blendor. Half diets were analyzed periodically in the laboratory. The composition of a typical liquid diet is given in Table II. Three to five days of *ad lib.* diet were allowed between diet study periods. All diets were supplemented daily with vitamin A 30,000 units, vitamin D 2,400 units, ascorbic acid 450 mgm., thiamine 12 mgm., riboflavin 18 mgm., nicotinamide 120 mgm., pyridoxine 7 mgm., folacin 3 mgm., and B_{12} 3 mcg. Dietary potassium ranged from 0.51 mEq. per gm. protein to 1.2 mEq. per gm. protein. The nitrogen balance technique used has been described (26). Body weights were determined daily, after voiding, before breakfast, on a scale accurate to $\pm$ 25 gm.

Statistical analyses of data were done using the methods of Fisher (27) and Ezekiel (28).

TABLE III

Nitrogen balance data

Patient number	Average body weight Kg.†	Daily caloric intake	Daily nitrogen intake *grams*	Daily stool nitrogen *grams*	Length of balance period *days*	Days used in calculating nitrogen balance	Average nitrogen balance *gm. nitrogen per day*
1	52.21	3,419	14.2	2.79*	11	Last 4	+2.77
2	45.45	3,550	17.3	2.53	14	Last 8	+7.22
	46.16	3,555	33.6	3.14	16	Last 8	+9.05
3	54.48	3,353	15.9	1.32	12	Last 7	+4.08
	56.76	3,352	31.3	1.77	12	Last 6	+7.44
	55.96	3,364	47.5	3.64	10	Last 3	+12.16
	54.30	2,598	16.2	1.31	9	Last 5	+3.96
4	48.87	3,000	31.7	3.11	11	Last 5	+7.30
	51.20	2,997	15.5	1.45	7	Last 4	+2.49
	54.31	2,998	27.5	3.91	10	Last 4	+3.86
5	44.93	1,995	15.7	3.12	8	Last 5	+3.31
	46.00	2,027	29.9	4.82*	7	Last 3	+4.36
	47.00	1,996	21.6	2.75*	10	Last 5	+4.29
	47.38	3,003	21.9	3.52*	12	Last 3	+4.54
	50.65	3,003	14.8	2.55	7	Last 4	+4.50
6	51.76	3,298	15.9	2.12	7	Last 4	+5.12
	55.78	3,299	28.2	2.06	11	Last 4	+6.02
7	52.10	3,144	17.5	1.80	13	Last 3	+2.68
	53.38	3,135	29.7	1.77	12	Last 6	+10.69
8	60.06	3,005	18.8	1.96	11	Last 5	+6.11
	61.89	3,004	44.5	6.95*	13	Last 6	+7.86
	61.93	2,999	31.4	3.73	14	Last 4	+5.67
9	47.90	3,493	39.3	2.60	14	Last 5	+6.06
	49.70	3,500	34.5	3.20	14	Last 6	+6.84
10	54.20	3,015	32.5	4.63*	14	Last 5	+7.40
	54.75	3,005	29.1	5.74*	12	Last 6	+6.18
	56.69	3,004	19.8	3.46*	10	Last 4	+2.86
11	51.17	3,715	27.1	2.28	8	Last 4	+8.94
12	48.57	2,816	7.8	1.56	8	Last 4	+2.86
13	51.27	2,851	8.7	1.25	8	Last 4	+1.93
	54.93	2,848	24.4	1.53	31	Last 7	+4.20

* Diarrhea during this stool collection period.

† The average of daily body weights on the days used for nitrogen balance calculation.

RESULTS

When the dietary nitrogen is changed a variable time is required for adjustment of body processes to the new nitrogen intake. Characteristically, the first day or two after a higher nitrogen intake is begun the nitrogen balance is more positive than is the case after a few days on the diet. In order to select days which most accurately represent the nitrogen balance, the present data were analyzed, as represented in Figure 1, to determine the time needed to reach a steady urinary nitrogen excretion at three ranges of nitrogen intake. In each case the preceding *ad lib.* diets provided nitrogen intakes estimated to be from 0.2 to 0.25 gm. N per Kg. per day. It can be seen that with diets containing 0.6 gm. or more N per Kg. per day the urinary nitrogen excretion continued to rise through the fourth day of the new diet and then levelled off. With diets containing 0.4 to 0.59 gm. N per Kg. per day, urinary nitrogen was relatively constant after the second day of the new diet. Urinary nitrogen excretion was nearly constant from the start with diets containing 0.16 to 0.39 gm. N per Kg. per day, although the variability was greater during the first two days.

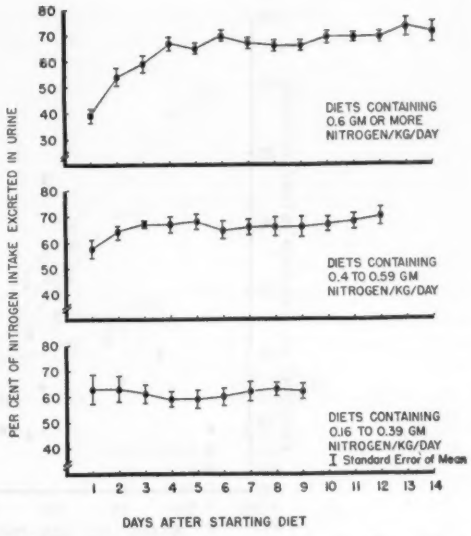
PER CENT OF NITROGEN INTAKE EXCRETED IN URINE
DIETS CONTAINING 0.6 GM OR MORE NITROGEN/KG/DAY
DIETS CONTAINING 0.4 TO 0.59 GM NITROGEN/KG/DAY
DIETS CONTAINING 0.16 TO 0.39 GM NITROGEN/KG/DAY
I Standard Error of Mean
DAYS AFTER STARTING DIET

FIG. 1. PER CENT OF NITROGEN INTAKE EXCRETED IN THE URINE WITH THREE RANGES OF DIETARY NITROGEN, AS A FUNCTION OF TIME AFTER STARTING DIET

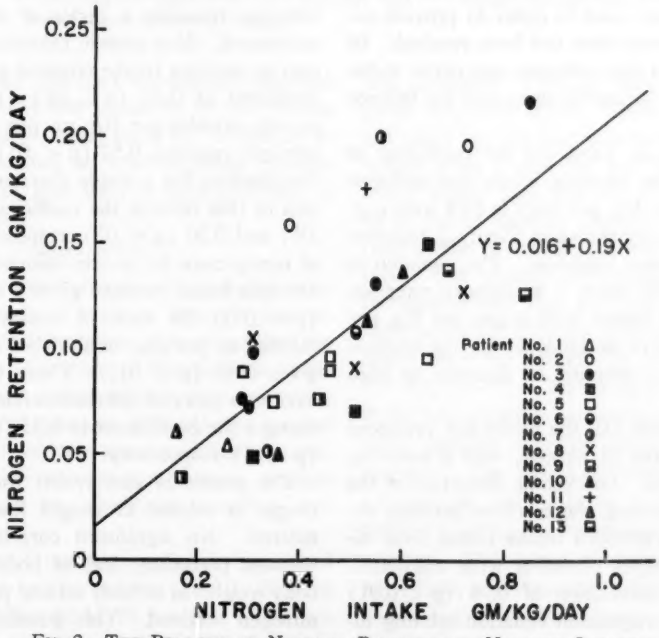

FIG. 2. THE RELATION OF NITROGEN RETENTION TO NITROGEN INTAKE

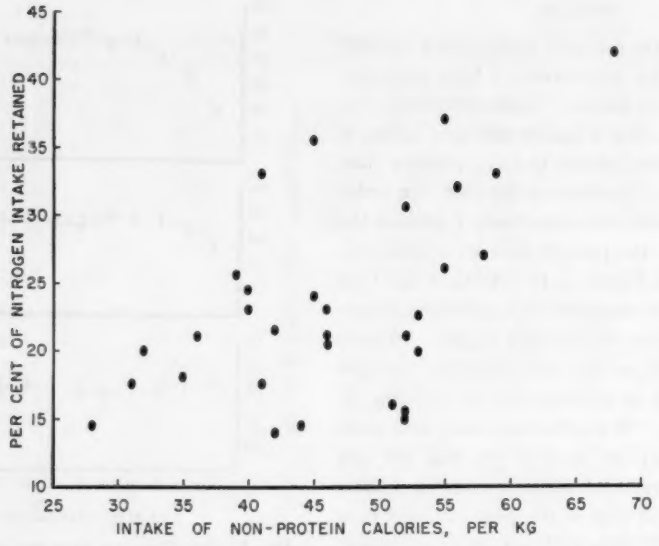

FIG. 3. THE RELATION OF NON-PROTEIN CALORIES TO PER CENT OF NITROGEN INTAKE RETAINED

In Table III are listed the results of the individual balance periods in the present study. In calculating nitrogen balances only the later days of each period were used in order to provide assurance that a steady state had been reached. In addition, the blood urea nitrogen was either stable or falling slightly during the days used for balance calculation.

From the data in Table III the coefficient of correlation between nitrogen intake and nitrogen retention (gm. per Kg. per day) is 0.74 with $p < 0.01$. These data are shown in Figure 2, together with their regression equation. The equation is $Y = 0.016 + 0.19X$ where Y is nitrogen retention and X is nitrogen intake, both in gm. per Kg. per day. The data show no tendency for the fraction of nitrogen intake retained to decrease at high levels of intake.

As noted in Table III, the liquid diet produced diarrhea in 8 of the 31 periods, with a resulting high fecal nitrogen. To exclude distortion of the results due to diarrhea, the relations between absorbed nitrogen (nitrogen intake minus fecal nitrogen) and nitrogen retention were calculated. A coefficient of correlation of 0.76 ($p < 0.01$) was found. The regression equation relating nitrogen retention (Y) and nitrogen absorbed (X), both in gm. per Kg. per day, was $Y = 0.019 + 0.21X$.

To assess the effect of non-protein calories on nitrogen retention a series of correlations were performed. Non-protein calories per Kg. *vs.* per cent of nitrogen intake retained gave a correlation coefficient of 0.53 ($p < .01$) (Figure 3); non-protein calories per Kg. *vs.* per cent of absorbed nitrogen retained, 0.52 ($p < .01$). Each of these distributions has a single aberrant value; elimination of this reduces the coefficients to 0.41 ($p < .05$) and 0.36 ($p = .05$), respectively. The ratio of non-protein to protein calories *vs.* per cent of nitrogen intake retained gives a coefficient of 0.39 ($p < .05$); the ratio of non-protein to protein calories *vs.* per cent of absorbed nitrogen retained gives 0.48 ($p < .01$). These two distributions have two pairs of aberrant values; their removal changes the coefficients to 0.35 ($p < .1$) and 0.29 ($p < .2$), respectively.

The possibility that avidity for retention of nitrogen is related to weight loss was also considered. No significant correlation was found between percentage loss of body weight, of lean body weight, or of body fat and per cent of dietary nitrogen retained. This possibility is also considered in Figure 4, in which are plotted individual

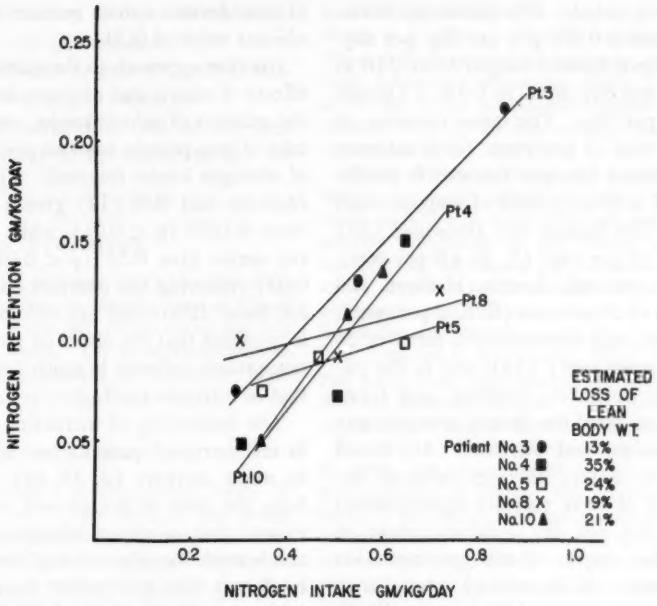

FIG. 4. RELATION OF NITROGEN RETENTION TO NITROGEN INTAKE IN EACH SUBJECT WHO RECEIVED THREE LEVELS OF DIETARY NITROGEN AT CONSTANT CALORIC INTAKES

No relation appears to exist between rate of nitrogen storage and estimated loss of lean body weight.

relations between nitrogen intake and nitrogen retention, drawn by best visual fit, for all patients with three isocaloric study periods. It appears in this small series that no relation exists between estimated loss of lean body weight and rate of nitrogen storage.

DISCUSSION

Patients with malnutrition are valuable subjects for the study of rates of nitrogen storage, since, for practical purposes, they can store unlimited amounts of nitrogen. The patients in the present series are a group with as near to uncomplicated malnutrition as may be found in a hospital population. All subjects were well into the anabolic phase of convalescence; all major wounds were healed and no significant infection was present.

Early studies of the response of malnourished men to various protein intakes (8–10, 20) led to the estimate that 60 to 70 per cent of the nitrogen intake was retained if the caloric intake was adequate. Five-day balance periods on any given diet were usual. These short periods did not allow for the lag in urinary excretion of nitrogen when a new diet was begun, and as a result a falsely high quantity of nitrogen appeared to be stored on high nitrogen intakes. In addition, as the protein intake was raised, the calorie intake was usually increased also; such caloric increments may also have tended to give falsely high values for nitrogen storage. The same criticisms may be directed at more recent studies (11–13, 16–21). The length of balance periods in the present study was sufficient to allow a stable urine nitrogen excretion and a stable blood urea nitrogen. The results so obtained permit the calculation of the relation, nitrogen retention = 0.016 + 0.19 times nitrogen intake, in gm. per Kg. per day. The values of nitrogen retention calculated from this equation include 28 of the 31 observed values within limits of ± 0.05 gm. per Kg. per day. Limits of ± 0.075 gm. per Kg. per day

include all but one value. The maximum deviation from predicted is 0.078 gm. per Kg. per day. The dietary nitrogen intakes ranged from 0.16 to 0.85 gm. per Kg. per day, equal to 1.0 to 5.3 grams protein per Kg. per day. The mean retention of dietary nitrogen was 23 per cent. This estimate of 23 per cent dietary nitrogen retained is similar to those obtained in other studies of malnourished adults. Strang, McClugage, and Brownlee (29) found a mean of 19 per cent (S. E. 4.0 per cent) dietary nitrogen retained, Beattie, Herbert, and Bell (12) a mean of 20 per cent (S. E. 2 per cent), Burger, Sandstead, and Drummond a mean of 24 per cent (S. E. 4 per cent) (13), and in the patients of Browne, Schenker, Venning, and Johnson (30) about one-third the dietary nitrogen was retained. Widdowson and Thrussell (31) found 40 per cent of the dietary nitrogen retained, but the caloric intake of their patients approximated 100 calories per Kg. per day, a caloric intake so high that a higher degree of nitrogen retention might be expected. Malnourished adults with chronic nephritis (32) retained 22 per cent (S. E. 1.2 per cent) while malnourished children (5) retained 14 per cent (S. E. 1.8 per cent) of the dietary nitrogen. Thus a substantial body of evidence indicates a relatively constant relation of nitrogen retention to nitrogen intake over a wide range of nitrogen intake in malnourished humans who are ingesting adequate calories. Contrary to the findings of Von Hoesslin (9) there is no indication of less efficient use of protein as the protein intake increases.

Burger, Sandstead, and Drummond (13) and Beattie, Herbert, and Bell (12) have stated that the amount of nitrogen retention in malnourished men is related more specifically to calorie intake than nitrogen intake. The data used as the basis of the belief in the pre-eminent influence of calories on nitrogen metabolism are presented in Table 5 of Beattie, Herbert, and Bell's paper (12). However, using these data, correlation of the per cent of absorbed nitrogen retained with the ratio of non-protein calories gives a correlation coefficient of -0.27 ($p > 0.1$). This negative correlation is due to the results in the patients of Group II where nitrogen and caloric intake were much lower than Group I and, as stated by the authors, may have been so low that any effect by non-protein calories could not be manifested. The correlation coefficient between these two measurements in the present series was 0.48 ($p < 0.01$); removal of two deviate values reduces it to the non-significant value of 0.29.

Another approach to the question of the relative effects of caloric and nitrogen intakes is to analyze the relation of caloric intake, more specifically intake of non-protein calories per Kg., and per cent of nitrogen intake retained. The data of Beattie, Herbert, and Bell (12) give a correlation coefficient of 0.79 ($p < 0.01$), while those of the present series give 0.53 ($p < 0.01$), or 0.41 ($p < 0.05$) removing the aberrant value. The reasons for these differences are unknown. However, it is apparent that the effect on nitrogen retention of non-protein calories is much less predictable than that of nitrogen itself.

The desirability of increasing nitrogen retention in malnourished patients has been commented on by many workers (2, 33, 34). It would appear from the data of the present series and that reviewed that increased nitrogen retention may be much more readily accomplished by an increase in dietary nitrogen rather than an increased caloric intake, provided caloric intake is adequate initially. A calorie intake of 35 calories per Kg. per day is considered adequate for patients comparable to those of the present series (12, 34).

The proposal that the degree of body weight loss determines the avidity for nitrogen in recovery from malnutrition was advanced by Rubner (35), and subsequently discussed by Wang, Hawks, and Hays (17). If the data of Wang, Hawks, and Hays (17) are analyzed statistically, no significant difference is present in the amount of nitrogen retained by the various groups studied with different degrees of underweight. Strang, McClugage, and Brownlee (29) found no relation between the rate of nitrogen storage and the degree of undernutrition of the patient, but Murray (36) found a more rapid weight gain in the more severely malnourished patients, although no nitrogen balance data are available in the latter instance. The data from the present series would indicate no apparent parallelism between estimated loss of lean body mass and rate of nitrogen storage.

CONCLUSIONS

1. Nitrogen retention is directly related to nitrogen intake in malnourished adults on adequate caloric intakes.

2. With nitrogen intakes ranging from 0.16 gm. to 0.85 gm. N per Kg. per day no diminished ef-

ficiency in the use of dietary nitrogen is evident at the higher levels of nitrogen intake.

3. Non-protein calories appear to have a relatively small effect on nitrogen retention.

4. In the present series the rate of nitrogen storage does not appear to be related to the degree of malnutrition.

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CASE SUMMARIES

Patient No. 1, a 20-year old infantryman, height 67 inches, average pre-injury weight 65 Kg., was struck by mortar fire in August 1951, receiving compound comminuted fractures of the right humerus and left femur and multiple penetrating wounds of the right foot and left leg, requiring amputation of the left leg. A long series of orthopedic and plastic surgical procedures were required in rehabilitation, the last, an open reduction of the right humerus, was done in December 1952. During this period, the patient also suffered three relapses of malaria due to *P. vivax.* When the study was begun in February 1953, the patient weighed 52.2 Kg. and appeared moderately underweight.

Patient No. 2, a 25-year old infantryman, height 72 inches, pre-injury weight 72 Kg., stepped on a land mine in December 1952, suffering compound comminuted fractures of both tibias and fibulas and the left ilium; and multiple penetrating wounds of both thighs and buttocks and the right forearm. The left leg was amputated during initial treatment; the right three months later, shortly before the start of the study. At that time, the patient weighed 44.5 Kg. and appeared markedly underweight. Anorexia, apathy, and marked lability of emotions were noted.

Patient No. 3, a 24-year old infantryman, height 67 inches, pre-injury weight 64 Kg., received a bullet wound in the left foot in November 1952, resulting in partial avulsion of the foot. After initial treatment, a series of reconstructive operations were done. At the beginning of the study in March 1953, the patient weighed 54.0 Kg. and appeared slightly underweight.

Patient No. 4, a 23-year old infantryman, height 71 inches, pre-injury weight 82 Kg., in October 1952 sustained multiple penetrating wounds resulting in compound comminuted fractures of the right tibia and fibula and left femur, right hemopneumothorax, and multiple superficial lacerations of the head and neck. Continuing treatment required amputation of the right leg and intramedullary nailing of the left femur. At the beginning of the study in May 1953, the patient weighed 46.0 Kg. Physical findings included moderate weight loss, healed right below-knee amputation stump, and a sinus tract at the site of the left femoral fracture draining purulent material from which was cultured hemolytic *Staphylococcus aureus* and *E. Coli.*

Patient No. 5, a 20-year old infantryman, height 68 inches, pre-injury weight 61.5 Kg., was wounded by small arms fire in April 1953 suffering avulsion of the anterior two thirds of the maxilla and hard palate, laceration of the tongue, and perforation of the sigmoid colon. Repair of the facial defect was begun at once; the perforated colon was exteriorized for ten days. Oral liquid diet was started a week after injury, but the patient continued to lose weight. When the study was begun eight weeks after injury, the patient weighed 45.3 Kg. and appeared markedly underweight. The abdominal and facial wounds were well healed.

Patient No. 6, a 23-year old tank commander, height 67 inches, pre-injury weight 66 Kg., was injured by a gun explosion in March 1953, receiving second degree burns of the hands, face, chest, abdomen and thighs; a compound comminuted fracture of the right ilium, penetrating wound of the liver with right hemopneumothorax and avulsion of the skin and superficial muscles of the left upper abdomen and left lower chest. Six weeks later at the start of the study, the patient weighed 51.0 Kg. and appeared moderately underweight. All wounds had healed except an area of clean granulation tissue 20×15 cm. over the right lower abdomen and upper thigh.

Patient No. 7, a 21-year old infantryman, height 70 inches, pre-injury weight 80 Kg., suffered bullet wounds in February 1953 resulting in multiple perforations of both large and small bowel, and severance of the left femoral artery. Amputation of the left leg above the knee was eventually necessary. At the beginning of the study in July 1953, the patient's wounds were healed, he weighed 49.5 Kg., and was moderately underweight.

Patient No. 8, 21-year old infantryman, height 69 inches, pre-injury weight 75 Kg., was injured by mortar fire in April 1953, receiving penetrating wounds of left side, including left arm, leg, abdomen, rupture of the spleen, laceration of the kidney, and fracture of the ilium. Recovery was complicated by thrombophlebitis. At the beginning of the study in June 1953, all wounds were healed and the thrombophlebitis had subsided. The patient weighed 58.8 Kg. and appeared moderately underweight.

Patient No. 9, a 17-year old infantryman, height 66.5 inches, pre-injury weight 62 Kg., suffered penetrating wounds of the skull, left eye, neck, left arm, right hip, and a compound comminuted fracture of the left tibia from a grenade explosion in May 1953. Treatment involved several craniotomies, a tracheotomy, and amputation of the left leg. When the study was begun in September 1953, the tracheotomy wound and amputation stump were not healing well. The patient weighed 44.9 Kg. and appeared moderately underweight.

Patient No. 10, a 22-year old infantryman, height 67 inches, pre-injury weight 68 Kg., suffered multiple penetrating wounds of both thighs with severance of the right femoral artery in July 1953. Primary anastomosis of the artery was successful. In September 1953, at the start of the study, all wounds were healed and only moderate weight loss was evident. The patient weighed 52.0 Kg.

Patient No. 11, a 22-year old infantryman, height 72 inches, pre-injury weight 66 Kg., sustained perforating wounds of the left cheek, right chest wall, and both legs in June 1953. Amputation of the right leg was necessary. At the start of the study in October 1953 the patient appeared moderately underweight and weighed 50.0 Kg.

Patient No. 12, a 22-year old infantryman, height 72 inches, pre-injury weight 75 Kg., received multiple fractures of the pelvis, with rupture of the bladder in August 1953. By the time the study was started in September 1953, all wounds were healed but the patient was markedly underweight. He weighed 47.7 Kg.

Patient No. 13, a 21-year old infantryman, height 69 inches, pre-injury weight 68 Kg., was wounded in March 1953, receiving a compound fracture of the left femur with laceration of the femoral artery and sciatic nerve. Treatment required a number of operative procedures; the course was complicated by hepatitis in September 1953. When the study was begun in October 1953 the patient weighed 50 Kg. and appeared moderately underweight. Liver function tests were normal. There was some purulent drainage from the site of the femoral fracture.

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INHIBITION OF URINE CITRATE EXCRETION AND THE PRODUCTION OF RENAL CALCINOSIS IN THE RAT BY ACETAZOLEAMIDE (DIAMOX®) ADMINISTRATION [1]

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We have previously observed that the urinary excretion of citrate is reduced to low levels in rats treated with the carbonic anhydrase inhibitor, acetazoleamide (Diamox®) (1). This first experiment was prompted by the fact that the elevated serum chloride and decreased serum bicarbonate concentrations, and the fixation of the urine pH at approximately 7 or above following administration of an agent which inhibits carbonic anhydrase activity resembles the findings in patients with congenital renal tubular acidosis. In a study of an infant with this syndrome we found that the urinary excretion of citrate was too low to be measured (1). The absence of citrate in the urine of this patient persisted even after the hyperchloremia and acidosis were corrected by administration of 40 mM of sodium lactate daily and the urine pH was raised to about 8.0. This is in contrast to the findings in normal subjects in whom the renal excretion of citrate increases when the urine is made alkaline by administration of sodium or potassium bicarbonate or the sodium and potassium salts of organic acids (2). It has also been shown that the administration of parathyroid extract (3) or vitamin D increases urinary output of citrate (4, 5). When these agents are given there is often a concomitant rise of urinary calcium excretion but the urinary output of citrate and of calcium do not necessarily parallel each other. Since citrate forms an undissociated complex with calcium the possibility that it is important in increasing the solubility of calcium salts in urine has been suggested (3).

TABLE I

Composition of diets (basic diet)

	gm.
Casein	180
Cornstarch	450
Dextrose	250
Peanut Oil	80
Brewer's Yeast	50
Salt Mixture	50*
Vit. A. Conc.	10,000 units

* Two salt mixtures were used which provided the following compositions in the final diet.

	Ca	P	Ash
	mg./gm.		
Diet A	16	2	Alkaline
Diet C	7	5.6	Acid

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service.

METHODS

The importance of urine citrate as a complexing agent could be tested by means of the effect of acetazoleamide upon renal excretion of citrate. These studies were devised to determine whether the increased citraturia due to administration of alkalinizing salts or of vitamin D could be inhibited by acetazoleamide and whether reduction of urinary excretion of citrate following acetazoleamide might be associated with precipitation of calcium salts in the renal tubules and formation of renal calculi. Three to four-week-old male albino rats of the Sprague-Dawley strain were placed on purified experimental diets for three weeks. At this time, when they had reached a weight of about 100 gm., they were divided into two groups, one a control and the other acetazoleamide-treated. Two diets were used which differed in their content of Ca and P and in their acid-base ratio as summarized in Table I. When acetazoleamide was given it was incorporated in the diet in the ratio of 250 mg. of drug per 100 gm. diet. The average daily dosage calculated from food intake was approximately 15 to 20 mg. per 100 gm. of body weight. The basic diets did not supply vitamin D which was added as mentioned in the description of the separate experiments. The animals were kept in metabolism cages permitting collection of urine without contamination by feces or spilled food and the urine was collected under toluene. Citrate in serum and urine was determined by the method of Natelson, Pincus, and Lugovoy (6). Calcium in urine was determined by the Clark-Collip modification of the Kramer-Tisdall method (7), and in serum by this method or by a micro method developed in this laboratory (8). Phosphorus was determined by the Fiske and Subbarow technique (9). Urine pH was measured by indicators.

RESULTS

In Figure 1 is shown the effect of acetazoleamide upon the urinary excretions of calcium and citrate of a rat fed diet A which is an alkaline ash, high calcium, low phosphorus ration. Although no vitamin D was given, the urinary excretions of citrate in the control period are considerable because of the marked cation excess (urine pH approximately 8.0). The sharp drop in citrate excretion following acetazoleamide addition is evident. The urine citrate output returns to control levels when the drug is discontinued and decreases again when it is resumed. The urinary excretion of phosphorus on this diet is negligible and is not influenced by the drug. The urinary excretion of calcium in the control period is high and there may be some inconstant decrease following acetazoleamide. The precipitous fall in urine citrate after acetazoleamide feeding is a consistent finding as is shown in Table II, which summarizes the results in six rats on diet A, four acetazoleamide fed and two control animals. Table III shows the results of a similar experiment in two rats fed diet A to which had been added a mixture of sodium bicarbonate and citric acid which supplied 90 mM of extra sodium and 60 mM (11.5 gm.) of citric acid per 100 gm. of diet. The purpose of this addition of sodium citrate was to increase further urinary excretion of citrate with the production of maximally alkaline urine. The urine citrate excretion is raised by addition of sodium citrate to

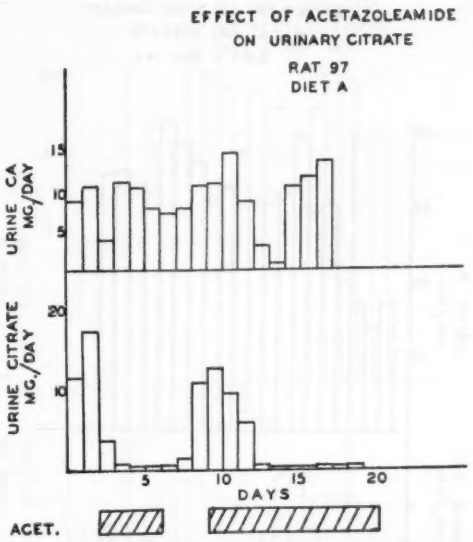


FIG. 1. EFFECT OF ACETAZOLEAMIDE ON URINE EXCRETION OF CITRATE AND CALCIUM OF RAT FED HIGH CALCIUM, LOW PHOSPHORUS, ALKALINE ASH DIET (DIET A)

Acetazoleamide added to diet during periods shown by diagonally striped bars at bottom of diagram.

TABLE II

Effect of acetazoleamide on urinary excretion of citrate by rats fed high calcium, low phosphorus diet

	Rat No.											
	122*		123*		124		125		126		127	
Day	Ca.	Cit.	Ca.	Cit.	Ca.	Cit.	Ca.	Cit.	Ca.	Cit.	Ca.	Cit.
	mg./day											
1	13.3		15.7		18.5		11.0		3.6		15.7	19.2
2	14.4	20.0	12.5	19.2	15.2	19.2	9.2	15.0	9.3	21.8	14.0	14.8
					Acetazoleamide added							
3	15.2		11.9									
4	11.8		11.5									
5	13.1	15.0	12.4	14.3	9.2	0.2	9.2	0.6	11.1		7.4	0.4
6	10.3	14.0	8.2		9.6	0.5	8.1		10.1	1.4	11.5	0.8
7	11.4		10.2		8.2	0.5	8.0	1.3	12.7	1.8	10.3	
8	7.2		11.7		6.0		4.2		9.1		6.2	
9	14.2		13.6		11.6		8.8		12.2		11.3	
10	12.4		12.7				6.7		8.0		6.1	
11	11.5	14.2	14.6	17.8	9.3	0.3	9.0	0.6	11.4	0.8	11.3	0.8
12	11.7		14.9		8.5		9.2		11.5			
13	10.1		14.6		9.4		8.0		11.5			
14	9.3	12.8	13.6	17.0	5.8	0.2	8.6	0.7	7.4	0.4		

* Control rats.

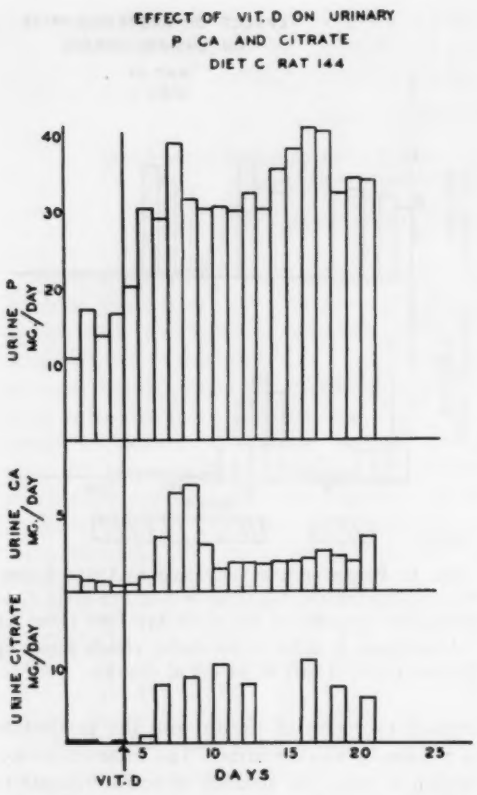


FIG. 2. INCREASE OF URINE CITRATE EXCRETION FOLLOWING VITAMIN D ADMINISTRATION TO RAT FED ACID ASH DIET, HIGH IN CALCIUM AND PHOSPHORUS (DIET C)

The arrow indicates day on which single dose of 5,000 units of vitamin D was given.

the high calcium, low phosphorus diet but the urinary excretion of calcium is reduced and urinary excretion of phosphate remains low. The inhibitory effect of acetazoleamide upon citrate excretion is still apparent despite the sodium citrate supplement although the urinary citrate is not reduced to the extremely low levels found in rats fed diet A without added sodium citrate. No evidence of refractoriness to the acetazoleamide effect on urine citrate excretion was found in rats treated continuously for three weeks. In a few experiments, doses of Diamox® of only 4 to 5 mg. per 100 gm. body weight per day were also effective in reducing urine citrate output to the same minimal values shown in Table II.

In Figures 2 and 3 are shown the effects of acetazoleamide upon vitamin D induced citraturia. In this experiment the animals were fed diet C in which the Ca to P ratio and the content of these elements per calorie of diet is approximately the same as in cow's milk. This is an acid ash diet and in the vitamin D deficient state the urinary citrate excretion is less than 0.5 mg. per day. The effect of the administration of a single dose of 5,000 units of vitamin D upon citraturia in a control rat is shown in Figure 2. The rapid increase of urinary citrate excretion to values of about 10 mg. per day following vitamin D is roughly paralleled by an increase in urinary excretion of both calcium and phosphorus. The increased urinary output of calcium and phosphorus presumably reflects increased intestinal absorption of these minerals due to vitamin D effect. Figure 3 shows a similar study in an acetazoleamide treated rat. The drug was added to the diet two days prior to the administration of vitamin D. The expected increase in citraturia due to vitamin D is almost completely prevented although the increased urinary output of calcium and phosphorus is approximately the same as in the control vitamin D treated rat. Similar results were obtained in three other rats on this regime. Urinary pH values ranged about 6.0 in the rats fed this diet without

TABLE III

Effect of acetazoleamide on urinary excretion of citrate by rats fed high calcium, low phosphorus diet supplemented with sodium citrate

	Rat No.			
	106		107	
	Ca	Cit.	Ca	Cit.
Day	mg./day		mg./day	
1	0.2	12.0	2.0	29.4
2	0.3	13.8	1.3	30.1
Acetazoleamide added				
3	0.2	2.0	3.4	27.5
4	0.9	1.6	6.7	13.4
5	0.6	2.7	3.4	11.7
6	0.6	5.5	1.9	6.1
Acetazoleamide discontinued				
7	1.0	5.9	3.0	10.7
8	0.4	6.9	2.0	18.0
9	1.7	17.5	0.9	25.2
10	1.0	23.5	2.1	24.4

acetazoleamide and were about 0.5 pH unit higher in the acetazoleamide-fed rats.

If urine citrate plays an important role in maintaining urinary calcium in solution as has been suggested by Kissin and Locks (10), Shorr, Almy, Sloan, Taussky, and Toscani (3), and Scott, Huggins, and Selman (11) the inhibition of citraturia by acetazoleamide without proportionate decrease of urine calcium excretion should conduce to the precipitation of calcium salts in the urinary tract. Six rats of the group fed diet C were given a single dose of 5,000 units of vitamin D. Four were fed acetazoleamide for a period of three weeks and two were maintained on the control diet. At the end of a three-week period the rats were sacrificed and blood samples and the kidneys were taken for analysis. The animals were not fasted and the treated rats received the Diamox® containing diet up to the time they were sacrificed.

The concentrations of calcium, phosphorus, and citrate in the serum of these animals are given in Table IV. The serum citrate values are essentially the same in control and treated rats despite marked differences in urinary excretion of citrate. Three additional control rats on this dietary regime had serum citrate concentrations of 3.0, 3.3 and 3.7 mg. per 100 cc., respectively. The serum calcium and phosphorus levels of both control and treated rats are all in the normal range for rats of this age although the serum phosphorus values are slightly higher in the treated animals. Half of each kidney was taken for chemical analysis and half for histological examination. The kidneys were ashed and calcium determined in the ash solution. The calcium contents of the kidneys are

TABLE IV

Serum citrate, calcium and phosphorus levels in control and acetazoleamide-fed rats, diet C *

Rat No.	Serum		
	Citrate	Ca	P
		mg./100 cc.	
143	4.4	10.2	9.0
144	3.5	10.2	8.8
145†	3.9	10.1	10.1
146†	4.0	9.6	9.6
147†	3.7	10.0	9.5
148†	3.3	10.2	10.6

* All rats had received a single dose of 5,000 units of vitamin D three weeks before they were sacrificed.
† Acetazoleamide added to diet.

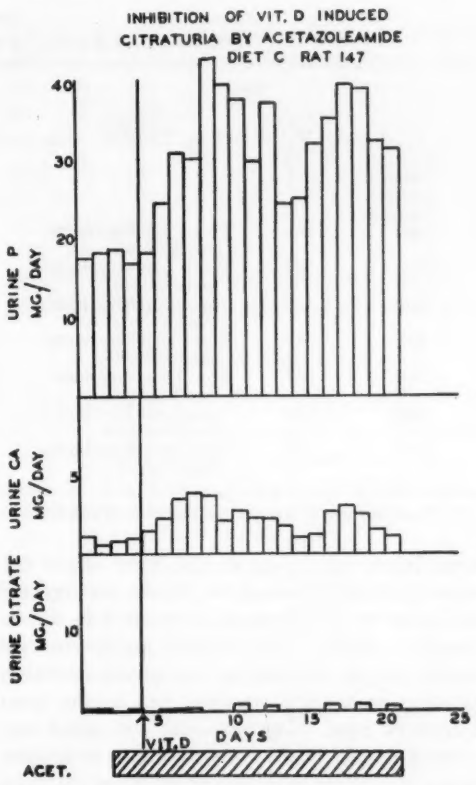


FIG. 3. INHIBITION OF VITAMIN D EFFECT ON URINE CITRATE AS RESULT OF ACETAZOLEAMIDE FEEDING

Period of acetazoleamide treatment shown by diagonally striped bar. The arrow indicates day on which single dose of 5,000 units of vitamin D was given.

shown in Table V. The kidneys of the two control animals contained too little calcium to be determined accurately and also showed no evidences of calcium precipitate on histological examination. The kidneys of the four acetazoleamide-fed rats contained between 27 and 355 mg. of calcium per 100 gm. fresh kidney. The location of the calcium deposits in the kidneys was determined by histological examination. The sections in Figure 4 stained by the von Kossa method show precipitated calcium in the kidney tubules chiefly at the cortico-medullary junction. The precipitated material is intratubular although the tubules are in many places disrupted by the precipitate. There is no histological evidence of

TABLE V

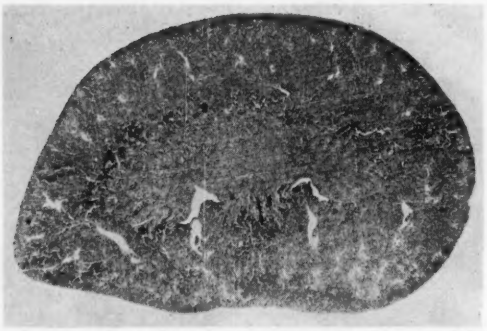
Calcium content of kidneys of control and acetazoleamide-fed rats

Diet A			Diet C		
Group	Ca *mg./100 gm.*	Visible calcification	Group	Ca *mg./100 gm.*	Visible calcification
Control	Trace*	0	Control	Trace*	0
Control	9	0	Control	Trace	0
Control	1	0	Acet.	113	Intratubular in outer zone of medulla
Control	3	0			
Acet.	105	Tips of papillary ducts; renal calculi	Acet.	217	Intratubular in outer zone of medulla
Acet.	46	Tips of papillary ducts; renal calculi	Acet.	27	Intratubular in outer zone of medulla
Acet.	51	Tips of papillary ducts; renal calculi	Acet.	355	Intratubular in outer zone of medulla
Acet.	29	Tips of papillary ducts; renal calculi			
Acet.	87	Tips of papillary ducts; renal calculi			
Acet.	96	Tips of papillary ducts; renal calculi			
Acet.	59	Tips of papillary ducts; renal calculi			

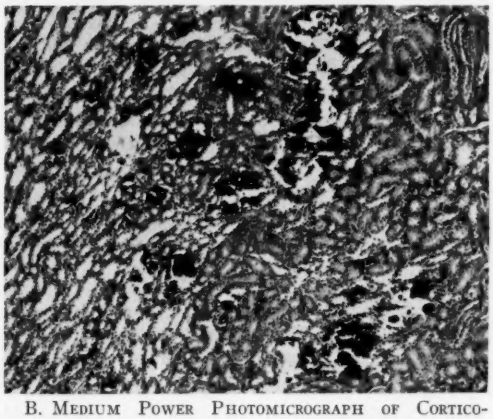
* Amount insufficient for quantitative determination.

renal tubule cell injury except at the site of calcium precipitation where the tubules are ruptured and there is no inflammatory reaction in the interstitial tissue. The calcium precipitates are found sharply localized at the cortico-medullary junction with some scattered foci in the inner medullary zone. The proximal and distal convoluted tubules in the cortex are free of calcium, as are the collecting tubules in the tip of the pyramid. The intratubular calcium precipitates are even more strikingly shown in the specimen illustrated in Figure 5. The kidney of an acetazoleamide-treated rat was split along the longitudinal axis and one-half was cleared by treatment with potassium hydroxide solution. The calcium was then stained with alizarin red. The calcium precipitates form casts of the tubules starting abruptly at the cortico-medullary junction and only a relatively short portion of the tubule is involved as is shown in the higher magnification (Figure 5B). The histological pattern suggests that most of the precipitation of calcium is in the terminal or straight segment of the proximal convoluted tubules. There may also be some precipitate in Henle's loops and in collecting tubules.

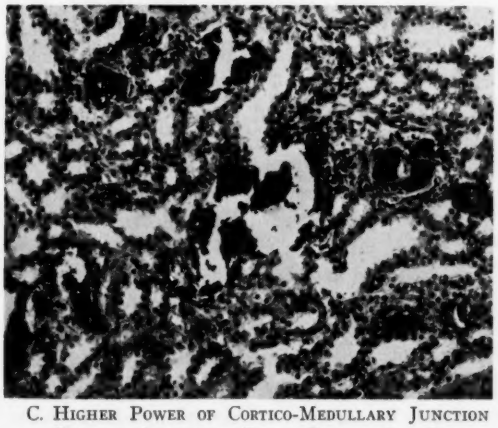
Similar studies were made on the kidneys of rats receiving the high calcium, low phosphorus diet without vitamin D supplement (diet A). The calcium contents of the kidneys of these rats are also given in Table V. The kidneys of the rats not receiving acetazoleamide showed only small amounts of calcium although the urine of these animals contained high concentrations of calcium. The kidneys of the control rats were normal both on gross and microscopic examination. Analyses of the kidneys of the acetazoleamide-fed rats showed considerable quantities of calcium. The pathological findings in these kidneys, however, differed from those seen in the kidneys of the rats fed diet C. On gross examination calculi of various sizes were found in the renal pelvis adherent to the renal papilla in almost all of the kidneys of the acetazoleamide-treated group. In one animal a calculus, weighing about 5 mg. had broken off and lodged in the bladder. This calculus was soluble in dilute hydrochloric acid and on analysis was a calcium stone, but no phosphate, citrate, oxalate, or sulfate was found. It was thought to be chiefly a calcium hydroxide, carbonate concretion. In two rats ureteral obstruction and hydronephrosis were found. On microscopic examination of the kidneys laminated masses of calcium precipitate were seen at the tips of the papillary ducts covered by the papillary epithelium as shown in Figure 6. Scattered foreign body giant cells were present at the periphery of these masses. No calcium precipitate was seen in any other portion of the kidney, however, although a deeply stained eosinophilic coagulum was found in many



A. Low Power Photograph Showing Calcium Deposits at the Cortico-Medullary Junction



B. Medium Power Photomicrograph of Cortico-Medullary Junction Showing Intratubular Calcium Precipitate in Distended Thin-Walled Tubules



C. Higher Power of Cortico-Medullary Junction Showing Absence of Renal Tubule Cell Injury Except for Mechanical Disruption of Tubules, and Lack of Tissue Reaction in Interstitial Tissue

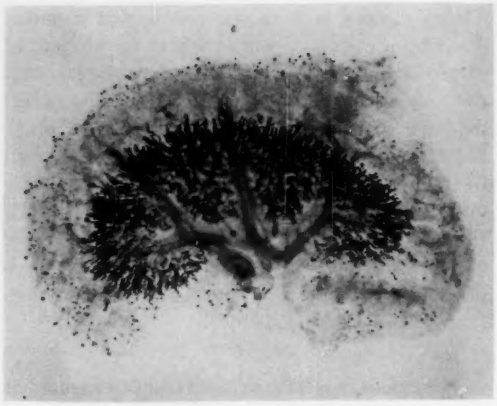
dilated tubules both in the cortex and medulla. The site of calcium precipitation in the kidneys of acetazoleamide-treated rats therefore differs with the type of diet fed and this effect of diet is apparently related to the concentration of phosphate in urine. The effect of acetazoleamide in the rats fed diet A was not altered by administration of vitamin D. The degree and site of calcium precipitation remained the same. In the rats fed this diet the urine phosphate excretion is practically nil with or without vitamin D feeding.

DISCUSSION

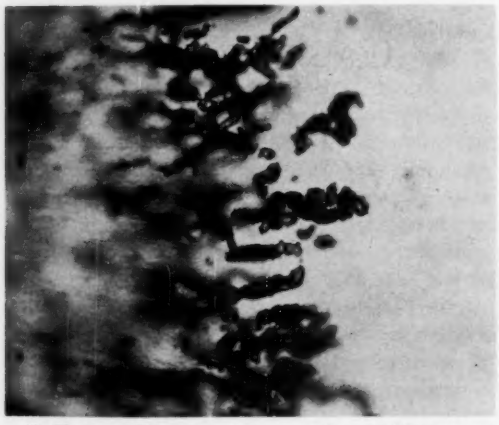
The mechanism of renal excretion of citrate has not been comprehensively studied but calculation of the clearances of this ion indicate that ordinarily most of the filtered citrate is reabsorbed (12). The effect of vitamin D in increasing urinary citrate might be due to increase in the amounts filtered since plasma citrate levels are increased by vitamin D (4). The excretion of citrate drops with decrease of urine pH and rises with increase of urine pH when the pH is altered by administration of acidifying or alkalinizing salts. This relationship to pH also holds true for other organic acids whereas the excretion of organic bases decreases as urine pH rises (12). It has been suggested that the increased urinary citrate excretion with cation excess is a buffer mechanism analogous to the increased excretion of ammonia under conditions of anion excess (2). Shorr, Carter, Smith, and Taussky (13) reported preliminary observations on the mechanism of citrate excretion in man. On the basis of simultaneous citrate and inulin clearances in a patient with Paget's disease they suggested that the increased citraturia due to alkali, and the decrease in citrate excretion due to testosterone administration resulted from changes in tubular reabsorption of citrate.

Acetazoleamide reduces urine citrate excretion despite its effect in increasing urine pH and it also prevents the increase of urine citrate output following vitamin D administration. The plasma citrate levels of acetazoleamide fed rats are the same as those of control rats fed a similar diet without acetazoleamide so that the differences in

Fig. 4. Kidney Sections of Acetazoleamide-Treated Rat Fed Diet C; Stained by von Kossa Silver Nitrate Method with Hematoxylin and Eosin Counterstain



A. Low Power of Entire Hemi-Kidney Showing Intratubular Calcium Casts Beginning at Cortico-Medullary Junction



B. Higher Magnification of Cortico-Medullary Junction Showing Intratubular Calcium Precipitate Beginning Sharply at Cortico-Medullary Border and Extending Only a Short Distance into the Medulla

Fig. 5. Kidney of Acetazoleamide-Treated Rat Fed Diet C

Specimen cleared by treatment with potassium hydroxide solution and the calcium stained with alizarin red.

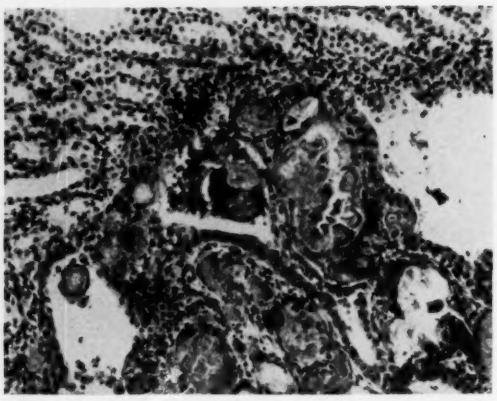


Fig. 6. Section of Kidney of Acetazoleamide-Treated Rat Fed Diet A; Section Taken at Edge of Papilla; Stained by von Kossa Silver Nitrate Method and Hematoxylin and Eosin

Laminated masses of calcium precipitate at tips of papillary ducts covered by papillary epithelium. Foreign body giant cells can be seen at periphery of these masses.

urinary citrate excretion must presumably be explained in terms of renal tubular activity. How this effect is related to the inhibition of carbonic anhydrase activity by acetazoleamide and to its postulated effect in reducing secretion of H^+ ions in exchange for Na^+ and K^+ ions (14) can only be conjectured. The intracellular pH of renal tubule cells might possibly be the factor controlling the excretion of citrate rather than the pH of tubular contents.

Whatever the mechanism which may be involved in the reduction of urinary citrate by acetazoleamide the sequence of events observed, namely, increase of urinary pH and decrease of urinary citrate without proportionate decrease in calcium or phosphate excretion evidently predisposes to precipitation of calcium in the renal tubules or in the upper urinary tract. These findings support the idea that urinary citrate does increase the solubility of calcium in urine by virtue of its property of forming an undissociated calcium complex and that this mechanism is of importance in the prevention of calcium precipitation in the kidney. The molar ratio of calcium to citrate in the urine of the control rats not receiving acetazoleamide ranged from 3:1 to 3:2. No studies were made to determine whether there might be other polycarboxylic acids excreted in the urine which could sequester calcium.

The site of precipitation of calcium in the urinary tract in these experiments is apparently determined by the concentration of phosphate in the urine. In both experiments the diets were such as to produce calcuria although the urine calcium

excretion was greater in the rats fed diet A. In the animals fed diet C supplemented with vitamin D the serum phosphorus concentrations are high and the phosphate load exceeds the renal tubular capacity to reabsorb phosphate so that the urine excreted contains phosphate. Under these conditions phosphate may be assumed to be present in the fluid leaving the proximal convoluted tubules. In the acetazoleamide-treated rats of this series calcium phosphate is precipitated in the renal tubules probably as the result of disappearance of citrate and also increase of pH of the tubular contents. The localization of the precipitate is of theoretical interest since the acetazoleamide effect upon citrate concentration or urine pH must presumably be exerted proximal to or in the portion of the tubule where precipitation of calcium phosphate occurs. The histological pattern of involvement of the tubules at the cortico-medullary junction, and the configuration of the tubular casts in the cleared specimen suggests that the precipitate is in the terminal or straight segment of the proximal convoluted tubule. Isolation of single nephrons by micro-dissection would be necessary to demonstrate conclusively the segment of the tubule in which precipitation occurs.

The feeding of a high calcium, low phosphate diet reduces the serum phosphorus level and therefore the phosphate filtered so that practically all of the phosphate filtered is reabsorbed in the proximal tubule. In the absence of phosphate in the tubular contents no calcium precipitation occurs in the upper nephron despite lack of citrate. Precipitation is found only at the tips of the papillary ducts with formation of calculi in the renal pelvis. Decrease in CO_2 tension with increase of pH or the physical factors of eddy currents and stasis may be involved in the precipitation of calcium from a supersaturated solution at this site.

The modifying effect of dietary factors upon precipitation of calcium in the kidney may explain the fact that such calcification has not been found by other workers who have been investigating acetazoleamide effect upon kidney function. Maren, Mayer, and Wadsworth (15) did note renal calcification in a few rats receiving very large doses of Diamox®, 900 mg. per Kg. No reports of renal calculus formation in patients receiving Diamox® have been published. The dosages given to patients in terms of body weight are much smaller than those used in the rat but the amounts used in these experiments are considerably greater than those needed to inhibit urine citrate excretion. Studies of the effect of Diamox® upon the urinary excretion of citrate, calcium, and phosphate in human subjects are needed before any conclusion can be drawn concerning the possibility of the development of renal calcinosis in patients receiving this drug. The renal calcification found in patients with renal tubular acidosis (16) might indeed be associated with an abnormality of urine citrate excretion in view of the extremely low urine citrate levels found in an infant with this syndrome.

SUMMARY

Administration of acetazoleamide to rats results in reduction of urinary citrate excretion to extremely low levels. The usual effect of cation excess or of vitamin D in increasing citraturia is inhibited by acetazoleamide.

The feeding of acetazoleamide to rats also causes the precipitation of calcium salts in the kidney or lower urinary tract under certain dietary conditions. On a diet which resembles cow's milk in its content of calcium and phosphorus, calcium phosphate is precipitated in the renal tubules at the cortico-medullary junction. If the diet is low in phosphate and high in calcium, calcified plaques are formed at the tips of the papillary ducts with formation of calculi in the pelvis. These findings indicate that the concentration of citrate in urine is of physiological importance in the stabilization of urinary calcium and that calcuria in the absence of urinary citrate results in precipitation of the calcium. The nature of the precipitate and its location in the renal tubule is apparently dependent upon the concentration of phosphate in the urine. The site of intratubular precipitation of calcium in the acetazoleamide-treated rats fed the high calcium and phosphorus diet suggests that the acetazoleamide effect upon citrate occurs in the proximal portion of the tubule.

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EFFECTS IN MAN OF ACUTE EXPERIMENTAL RESPIRATORY ALKALOSIS AND ACIDOSIS ON IONIC TRANSFERS IN THE TOTAL BODY FLUIDS [1]

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Although the concept of tissue buffering was formulated early in the study of acid-base disturbances, attention in the past has been directed primarily towards the effects of these disturbances on blood and extracellular fluid. More recently the demonstration by Darrow and his co-workers (1–3) of exchanges of intracellular cations during clinical and experimental potassium deficiency stimulated renewed interest in the quantitative aspects of the buffering action of intracellular fluid. Accordingly, some years ago the authors undertook quantitative estimation of the effects in man of acute experimental respiratory alkalosis and acidosis on the components of the multiphase system of red cells, plasma, interstitial fluid, and "intracellular" fluid (calculated as the non-chloride space), as well as on exchanges in the kidney. The latter renal effects will be reported in another paper (4).

Our results indicate that in man the effects of acute hyperventilation or carbon dioxide inhalation are buffered to a large extent by a series of linked ionic exchanges with a phase or phases outside the chloride space, as well as with buffer systems within the blood and extracellular fluid. Since these findings were reported in abstract (5–8) similar conclusions have been reached by Giebisch, Berger, and Pitts (9), on the basis of experimental respiratory disturbances in the dog. Estimates of the importance of tissue buffering have also been made in acute metabolic alkalosis and acidosis in man (10, 11) and in the dog (12–14).

EXPERIMENTAL PROCEDURE AND METHODS

The effects of the two opposite types of respiratory disturbance, hyperventilation and CO_2 inhalation, were assessed by comparison of the changes observed from the control period to the period during altered respiration and from the latter to the recovery period. This assessment has been made by use of the balance technic; the method of calculation is presented in the next section.

Twelve normal men between the ages of 19 and 34, students and physicians, served as experimental subjects. All subjects were in the fasting state and the studies were performed during the morning hours (8 A.M. to 12 noon); during this interval diurnal variations in acid-base balance and renal function should be small, unidirectional, and comparable between all experiments as a control background. Standard loading of the subjects with water and solutes was carried out before and during the experiments as follows. In the respiratory alkalosis experiments, this consisted essentially in water loading only in amounts equivalent to urine excreted. In the respiratory acidosis experiments, Experiments 1 to 5 inclusive, the subjects were given 4.2 gm. $NaHCO_3$ (50 mEq.) by mouth at -95 to -150 minutes to produce a very slight metabolic alkalosis and ensure a neutral or alkaline urine before the onset of the stimulus; in the sixth experiment NaCl was given as a control. In all the latter experiments one liter of water was given with the salt as a priming solution to institute an adequate and relatively constant urine flow; subsequently water was taken orally in amounts equivalent to urinary excretion. All subjects remained recumbent throughout the experiments.

Following control periods of 47 to 74 minutes' duration, *respiratory alkalosis* was induced by voluntary hyperventilation for approximately 30 minutes in 5 of the 6 experiments, and for twice that period in the last experiment; *respiratory acidosis* was induced by breathing 7.5 to 7.7 per cent CO_2 in air or O_2 for periods of 21 to 30 minutes. Observations were continued during recovery periods of 67 to 110 minutes in duration. During both sets of experiments serial measurements of the acid-base factors of blood and of the concentration of elec-

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trolytes in plasma were made on samples of arterial or cutaneous arterialized blood (15, 16). In a few instances ions other than bicarbonate were measured in venous serum. Simultaneously measurements were made of the renal clearance of electrolytes and of renal hemodynamics, to be described in a subsequent paper (4).

Heparinized blood for acid-base studies was collected and handled with precautions to prevent loss of CO_2 (10, 16). One portion of each sample was centrifuged anaerobically, and the plasma drawn off and stored in a greased syringe for analysis of total CO_2 by means of the manometric Van Slyke and Sendroy apparatus (17), sodium and potassium with a Barclay internal standard flame photometer (18), and chloride by a titrimetric silver iodate method (19). The remaining portion of each sample of blood was stored in the capped syringe in an ice bath until aliquots were taken for determination of hemoglobin (20), and of hematocrit value, pH and whole blood CO_2 by a modification of the Shock and Hastings microtechnique (21, 16). In this modification visual colorimetric comparison of pH at 37° C. was replaced by reading in the Klett-Summerson photometer, with a green filter, in a manner similar to that described by Van Slyke, Weisiger, and Van Slyke (22). The standard error of this measurement was ± 0.009 pH unit for a single reading; these measurements, however, were always made in triplicate. Venous blood, when used, was allowed to clot, was centrifuged under oil, and the serum was drawn off for analysis of sodium, potassium, and chloride. By use of the Singer-Hastings nomogram (15) and the Henderson-Hasselbalch equation,[5] values were derived (from the observed values of plasma pH as measured in whole blood and from CO_2 content of plasma) for three additional values: arterial CO_2 pressure, plasma bicarbonate concentration, and concentration of buffer base. Buffer base represents the cation equivalent of the sum of bicarbonate and all other buffer anions.

CALCULATIONS

General principles. Transfers of sodium and potassium often have been estimated in respect to calculated changes in "extracellular" fluid as equated with the chloride space (1, 24). In essentially the same manner it is possible to calculate from the changes in total "extracellular" bicarbonate the transfers of hydrogen ion into and out of the chloride space, when allowance has been made for the uptake or release of hydrogen ion by the non-bicarbonate buffers in red cells and plasma, and for the excretion of hydrogen by the kidney. The method of calculation employed here is similar to that described previously (10), but allowance is also made in the present report for shifts of chloride between red cells and plasma. This is necessary since under these experimental circumstances such shifts may make a significant difference in calculated fluid volumes. These calculations require values for initial and final "extracellular" fluid volumes. Such an initial volume was assumed and the final volume was calculated by correction according to change in serum concentration and external balance of chloride. This is probably at least as accurate for our purpose as direct measurement by substances such as inulin or radio-sulfate since the *changes* in this chloride space and in the associated cationic transfers are relatively little affected by variations in the initial *absolute* "extracellular" volume. The validity of the use of the chloride space is taken up under *Discussion.*

Transfers of hydrogen ion into and out of the chloride space, the red cells being included with this "extracellular fluid," may be calculated from changes in the total amount of reacting buffer anions. Hydrogen ion can combine with bicarbonate or non-bicarbonate buffers (Buf^-), chiefly hemoglobin and plasma protein anions in blood, in the following manner:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2 \qquad (1)$$

$$H^+ + Buf^- \rightleftharpoons HBuf \qquad (2)$$

The CO_2 is volatile, HBuf is non-volatile. In metabolic acidosis the influx of strong acid provides hydrogen ion which must be buffered by decrease of both bicarbonate and other buffer anions as shown in Equations *1* and *2*. The excess H^+ which reacts with dissociated buffer anions, Buf^-, produces an equivalent rise in the undissociated acid, HBuf, and this excess H^+ can be identified as remaining in the chloride space in the form of a non-ionized acid. On the other hand, the excess H^+ reacting with HCO_3^- ordinarily cannot be so identified, since the non-metabolic carbonic acid formed is broken down into water and carbon dioxide which is very promptly eliminated through the lungs. The excess H^+ therefore is accountable as a measured or calculated *decrease* in the sum of the buffer anions, or in their cationic equivalent, the buffer base, BB^+. In primary respiratory disturbances the respective changes in HCO_3^- and Buf^- are in opposite directions, instead of in the same direction. When whole blood is saturated *in vitro* with air-CO_2 mixtures each increase or decrease in HCO_3^- is balanced by an equal and opposite change in Buf^-. The reactions represented in Equations *1* and *2* are reciprocal reactions, and the buffer base content of whole blood is constant, although the chloride shift results in a limited redistribution of the buffer base between plasma and red cells.

The situation is more complicated when such changes are considered *in vivo*, with at least four phases to be accounted for simultaneously. These include red cells, plasma, interstitial fluid, and "intracellular" fluid. In addition, effects of renal excretion must be considered. By means of appropriate measurements all of these factors can be calculated in such a way as to yield a quantity designated the "intracellular" hydrogen change, ΔH_i^+.

[5] From the Henderson-Hasselbalch equation:

$$pH = pK_1' + \log \frac{(HCO_3^-)}{fP_{CO_2}},$$

where

$pK_1' = 6.10$ at 37° C. (23)

f = 0.0314 mM per liter per mm. P_{CO_2} at 37° C. in plasma or serum with an average water content of 938 g. per liter (23).

This value represents hydrogen ion that must be accounted for in respect to a change in the total quantity of "extracellular" plus red cell buffer base, ΔBB_{er}^{+}, corrected for the external non-respiratory balance of hydrogen ion, b_H^{+}. It is calculated as follows:

$$\Delta H_i^{+} = \Delta BB_{er}^{+} - b_H^{+}. \quad (3)$$

Although this is designated as hydrogen moving in one direction, it may also represent bicarbonate moving in the opposite direction across the phase boundary; the two cannot be differentiated. If ΔH_i^{+} is positive, one may refer to a transfer of hydrogen ion into the cells, and if negative, out of the cells. It should be understood, however, that these opposite changes may represent only a change in the rate of movement of the ion in a constant direction rather than reversal of its movement. The continuing metabolism present in the dynamic equilibrium which we designate as a "steady state" provides acid products which cause a steady movement of hydrogen ions out of the intracellular fluid. In response to respiratory acidosis one may calculate a positive ΔH_i^{+}, and describe it as a shift of hydrogen ion into cells. It may represent, however, a decrease in the steady rate at which the ion is moving out of cells, a process that would have an identical effect on the intracellular and extracellular fluid.

The method of calculation of ΔBB_{er}^{+} was similar to that described previously (10), with certain modifications occasioned by the fact that the chloride shift cannot be neglected in respect to these ionic transfers. The altered quantity of buffer base was derived from

$$\Delta BB_{er}^{+} = \Delta HCO_{3er}^{-} + \Delta Buf_b^{-}, \quad (4)$$

where ΔBuf_b^{-} is the change in non-bicarbonate blood buffer anions as calculated below in Equation *9*, and ΔHCO_{3er}^{-} is the change in total "extracellular" plus red cell bicarbonate calculated as follows:

$$\Delta HCO_{3er}^{-} = \Delta HCO_{3p}^{-} + \Delta HCO_{3f}^{-} + \Delta HCO_{3r}^{-}. \quad (5)$$

For plasma, interstitial fluid and red cells, indicated by subscripts p, f and r, respectively, the change in the amount of bicarbonate between observation 1 and *2* is given by the equation:

$$\Delta HCO_3^{-} = V_2(HCO_3^{-})_2 - V_1(HCO_3^{-})_1, \quad (6)$$

where V_1 and V_2 represent initial and final volumes, respectively. The bicarbonate concentration in plasma, $(HCO_3^{-})_p$, was calculated from the observed plasma total CO_2 concentration and pH, by means of the Henderson-Hasselbalch equation. Red cell "bicarbonate" (including carbamino CO_2) was calculated from the whole blood total CO_2, plasma total CO_2 and red cell volume, the appropriate subtraction being made for carbonic acid on the basis of the solubility coefficient and CO_2 pressure. The whole blood CO_2 concentration was calculated from the plasma CO_2 concentration, pH, and whole blood hemoglobin concentration by means of the line chart of Van Slyke and Sendroy (17), on the assumption of complete oxygen saturation in the arterial or cutaneous blood. In the experiments where cutaneous blood samples were used the plasma CO_2 concentration was calculated from whole blood CO_2. Interstitial fluid bicarbonate concentration, $(HCO_3^{-})_f$, was derived from $(HCO_3^{-})_p$ by the following equation:

$$(HCO_3^{-})_f = 1.16\ (HCO_3^{-})_p. \quad (7)$$

The factor 1.16 is based on a Donnan ratio of arterial plasma to interstitial fluid bicarbonate concentration, expressed per kilogram of water, of 0.91, and a water content of 935 grams per liter in plasma and 990 grams per liter in interstitial fluid (25). The rather low value of this Donnan ratio as compared with the usually quoted one of 0.95 or 0.96 is explained by the relatively large arterio-venous difference for bicarbonate. The corresponding factors which were used for the concentrations of Cl, Na and K were as follows: 1.08, 1.015, and 0.97, respectively (*26, 27*).

Initial control values of blood volume, V_b, and extracellular fluid volume, V_e, were assumed, 80 ml. per Kg. body weight for the former (*28, 29*) and 200 ml. per Kg. body weight for the latter (10). Successive values of blood volume were calculated from hemoglobin concentrations, (Hb), by the equation:

$$V_{b_2} = V_{b_1}\frac{(Hb)_1}{(Hb)_2}. \quad (8)$$

For each blood volume the corresponding plasma and red cell volumes, V_p and V_r, were derived with the aid of the observed hematocrit value. The initial interstitial fluid volume was obtained as the difference between the initial assumed extracellular and plasma volumes. Subsequent values of interstitial fluid volume, V_f, were calculated in terms of the chloride content of blood and interstitial fluid, allowance being made for loss of chloride in the urine and shifts between blood and interstitial fluid; the assumption was made that there was an equilibrium in chloride concentration between blood and interstitial fluid at the time of sampling. Where whole blood chloride was not measured an initial value was calculated and transfers between plasma and red cells were taken in accordance with the pH changes and the average transfer per unit change in pH observed in the arterial blood experiments. For each observation after the initial one it was thus possible to calculate (a) the amount of chloride in interstitial fluid, *on the assumption that no exchange occurs with tissue cells,* (b) the interstitial fluid chloride concentration, $(Cl^{-})_f$, as $1.08 \times (Cl^{-})_p$, and (c) the interstitial fluid volume as the quotient of amount divided by concentration. These volumes were used to calculate not only the bicarbonate distribution, but also the distribution of extracellular sodium and potassium.

The final term in Equation *4*, the change in non-bicarbonate blood buffer anions, was calculated from the observed pH changes, hemoglobin concentration, and assumed blood volume as follows:

$$\Delta Buf_b^{-} = [7.0\ V_p + 2.3\ (Hb)_b\ V_b]\ \Delta pH. \quad (9)$$

The hemoglobin concentration is here expressed in mM per L. and pH in units of $1 = 0.01$. This equation is based

on buffer data for normal human blood of Dill, Edwards, and Consolazio (23), as adapted in a slightly different form (15).

With the value for ΔBB_{er}^{+} thus obtained, the non-respiratory hydrogen ion balance, b_H^{+}, must be calculated in order to solve Equation *3* for change in intracellular hydrogen ion. In the absence of intake this hydrogen ion balance is calculated from the urinary excretion of ammonia and titratable acid, $UV_{NH_4}^{+}$ and UV_{TA}, which measure hydrogen excreted through the kidney, and of bicarbonate, $UV_{HCO_3}^{-}$, which is equivalent to hydrogen ion added to the body:

$$b_H^{+} = -[(UV_{NH_4}^{+} + UV_{TA}) - UV_{HCO_3}^{-}]. \quad (10)$$

Given the values for ΔBB_{er}^{+} and for b_H^{+} the change in intracellular hydrogen ion, ΔH_i^{+}, is then calculated from Equation *3*.

RESULTS

I. Effects on blood and plasma

All results have been evaluated by standard statistical procedures. These results are presented in Tables I A and I B and in Figures 1 and 2.

Acid-base factors of blood and plasma (*Figure 1*). In response to voluntary *hyperventilation* the

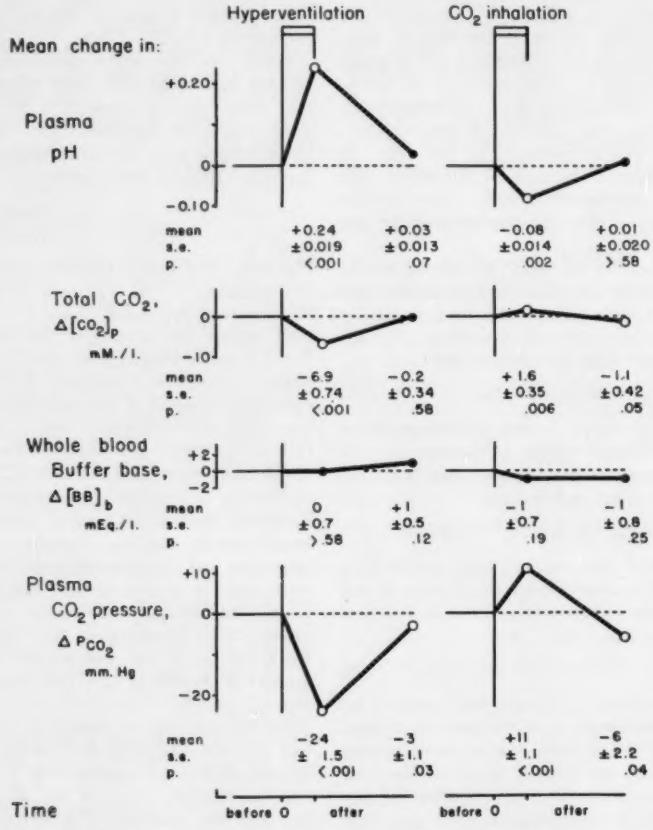

FIG. 1. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN PLASMA pH, PLASMA CO_2 CONTENT, WHOLE BLOOD BUFFER BASE CONCENTRATION, AND CO_2 PRESSURE IN PLASMA

The mean for each group of changes from the individual mean control values are plotted for the end of the period of stimulus and the end of the recovery period. The values for the mean, its standard error, and the probability that the mean change is significantly different from the control, are given below the curves. The mean changes that are statistically significant are represented by open circles.

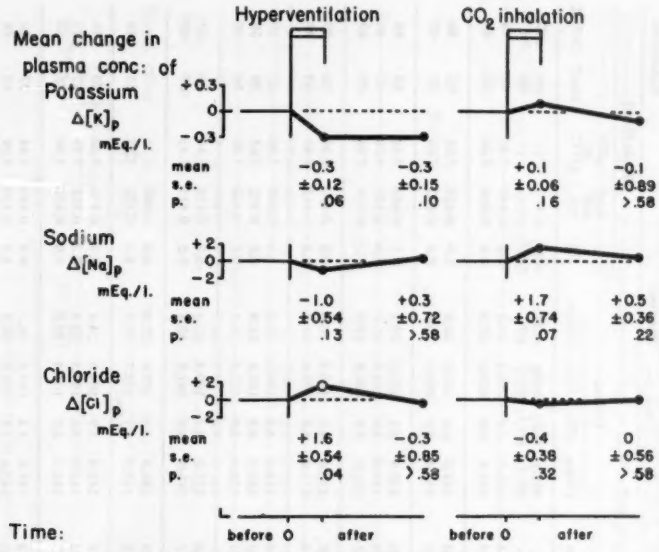

FIG. 2. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN THE PLASMA CONCENTRATION OF POTASSIUM, SODIUM, AND CHLORIDE
The data are presented as in Figure 1.

pH rose (mean = + 0.24 pH unit), the CO_2 content fell (− 6.9 mM per L.), the pressure of CO_2, P_{CO_2}, fell (− 24 mm. Hg), and the buffer base concentration, $(BB^-)_b$, was constant. These changes define a primary respiratory alkalosis or carbonic acid deficit. After the stimulus these values returned to or towards the control levels. In *CO_2 inhalation* the pH fell (− 0.08 pH unit), the CO_2 content rose (+ 1.6 mM per L.), the P_{CO_2} rose (+ 11 mm. Hg), and the buffer base concentration was essentially unchanged (− 1 mEq. per L. These findings are characteristic of a primary respiratory acidosis or carbonic acid excess. After the inhalation, elevated values for P_{CO_2} and total CO_2 returned to normal, actually overshooting to reach a significantly decreased value at the end of the experiments. Since the mean changes in pH and P_{CO_2} were greater during voluntary hyperventilation than during CO_2 inhalation, it is apparent that of the two procedures the former was a considerably more severe disturbance and hence, in comparison to CO_2 inhalation, evoked more extreme as well as more consistent responses in the body.

Plasma concentration of other electrolytes (Figure 2). Slight changes were obtained in the mean concentrations of sodium, potassium, and chloride. Although in most instances these changes were not statistically significant, they were in opposite directions with the two stimuli and in the direction predictable on theoretical grounds. When converted from concentration changes to changes in total extracellular amounts many of these changes are significant. The decrease in potassium concentration of − 0.3 mEq. per L. at the end of hyperventilation (significant at the 6 per cent level but not at the 5 per cent level) is in accord with other observations in experimental respiratory alkalosis (30, 31). The significant increase in chloride concentration, + 1.6 mEq. per L., occurring in respiratory alkalosis, is predicted on theoretical grounds from the effect of the pH rise in decreasing the Donnan ratio of red cell chloride to plasma chloride (23).

II. Calculated effects on ionic transfers in the total body fluids

These results are presented in Tables II A, II B, III A, and III B, and in Figures 3 to 6, inclusive. In Tables II A and II B are presented the observed balance data, the calculated ionic concentrations in

TABLE I A

Respiratory alkalosis: Effects of hyperventilation on arterial blood and plasma

Expt. and duration stimulus in min.	Time*	Observed: Plasma $(Na)_p$	$(K)_p$	$(Cl)_p$	$(CO_2)_p$	Observed: Whole blood Hb	Red cell vol.	pH† at 37° C.	Calculated P_{CO_2}†	Calculated $(BB)_b$‡
	min.	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mM. per L.*	*mM. per L.*			*mm. Hg*	*mEq. per L.*
1§	−36	—	—	—	27.0	9.8	0.485	7.42	40	50
	− 5	—	—	—	27.4	9.6	0.465	7.43	40	51
	+17	—	—	—	22.0	9.8	0.470	7.59	23	50
(29)	+29	—	—	—	19.8	10.2	0.485	7.61	20	48
	+61	—	—	—	26.3	9.85	0.485	7.43	39	50
	+117	—	—	—	26.1	9.8	0.470	7.41	40	49
3	−47	133.2	4.0	106.0	25.4	8.6	0.418	7.42	38	47
	− 4	136.0	4.1	106.5	26.9	8.5	0.408	7.33	48	46
	+12	137.0	3.8	108.5	20.5	8.5	0.410	7.62	19	48
(30)	+27	132.5	3.5	108.0	19.7	8.7	0.415	7.62	19	48
	+52	133.0	3.3	106.5	25.9	8.5	0.418	7.42	39	48
	+87	135.2	3.5	105.7	26.1	8.3	0.408	7.40	40	48
	+118	134.0	3.3	105.7	26.8	8.3	0.398	7.40	42	48
4	−64	133.0	3.7	107.6	24.5	9.7	0.493	7.34	43	45
	− 3	136.0	3.7	107.1	25.3	9.5	0.488	7.36	43	46
	+19	134.5	4.1	108.6	19.3	10.0	0.485	7.52	23	46
(37)	+35	132.0	3.6	108.4	16.8	10.2	0.490	7.65	15	46
	+60	136.5	3.4	105.6	23.3	9.8	0.500	7.42	34	46
	+83	136.0	3.8	105.8	24.9	9.8	0.495	7.43	36	48
	+114	133.5	3.6	105.2	25.7	9.8	0.493	7.43	37	48
5	−53	138.0	3.8	108.4	26.6	8.8	0.440	7.40	41	48
	− 4	139.0	3.9	109.0	27.8	8.8	0.440	7.38	45	49
	+13	141.5	4.4	110.0	21.6	8.9	0.435	7.61	21	50
(32)	+29	138.0	3.9	109.4	20.6	9.4	0.452	7.63	19	49
	+49	140.0	3.4	109.0	25.8	9.1	0.453	7.42	38	48
	+79	139.0	3.9	107.8	26.9	9.0	0.440	7.39	43	48
	+112	139.0	3.9	106.8	27.0	9.0	0.438	7.44	39	49
6	−56	135.0	4.3	100.4	26.0	8.2	0.400	7.34	46	46
	− 4	135.0	4.4	97.6	27.2	8.3	0.415	7.43	40	49
	+ 5	134.0	4.4	102.4	22.3	8.6	0.400	7.60	23	50
	+13	134.0	4.3	101.6	21.0	8.8	0.405	7.63	20	50
(27)	+27	135.0	3.9	102.6	18.1	9.1	0.425	7.66	16	48
	+48	135.0	3.7	100.4	25.4	8.7	0.425	7.44	36	49
	+112	138.0	3.9	101.4	25.5	8.5	0.415	7.43	37	48
7	−59	143.5	3.8	103.0	27.3	8.6	0.430			
	− 6	143.5	3.7	103.7	26.7	8.6	0.425	7.37	44	48
	+ 8	143.5	3.9	104.8	24.4	8.7	0.428	7.50	30	49
	+22	142.5	3.6	104.3	23.8	8.8	0.425	7.49	30	48
(58)	+52	143.5	3.6	104.3	23.0	9.0	0.435	7.56	25	50
	+72	145.5	3.4	103.8	26.1	9.0	0.440	7.44	37	49
	+141	143.0	3.5	104.0	26.2	8.5	0.420	7.39	42	48

* Time as indicated is measured from start of hyperventilation; values obtained during hyperventilation are separated by horizontal single lines.
† pH was determined on whole blood but is a measure of the hydrogen ion concentration of plasma; calculated P_{CO_2} is identical for both plasma and whole blood.
‡ $(BB)_b$ indicates whole blood buffer base concentration.
§ In Expt. 1 arterialized cutaneous, rather than direct arterial, blood was obtained and the plasma content of CO_2 calculated from that of whole blood.

TABLE I B

Respiratory acidosis: Effects of CO_2 inhalation on arterial or arterialized blood and plasma*

Expt. and duration stimulus in min.	Time†	Observed							Calculated	
		Plasma				Whole blood				
		$(Na)_p$	$(K)_p$	$(Cl)_p$	$(CO_2)_p$	Hb	Red cell vol.	pH‡ at 37° C.	P_{CO_2}‡	$(BB)_b$§
	min.	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mM. per L.*	*mM. per L.*			*mm. Hg*	*mEq. per L.*
1	−42	138.0	3.5	102.5	29.4	8.0	0.400	7.39	47	51.5
	−1	139.0	3.5	101.7	28.9	7.9	0.395	7.40	45	51.5
	+7	139.5	3.5	101.2	31.3	8.2	0.425	7.34	56	51
(30)	+30	143.5	3.5	101.1	30.9	8.2	0.420	7.35	54	51
	+35	143.0	3.5	100.0	29.4	8.1	0.410	7.41	45	51
	+93	140.0	3.4	100.7	28.5	8.0	0.405	7.45	40	52
2	−24	145.0	—	101.0	29.5	9.2	0.440	7.46	40	53
(27)	+18	146.0	—	101.5	30.2	9.2	0.435	7.40	47	52
	+63	145.0	—	102.5	28.0	9.2	0.440	7.43	40	51
3	−35	146.0	3.5	100.5	—	—	—	—	—	—
	−12	146.0	3.9	101.2	26.8	9.3	0.420	7.50	34	51
(29)	+17	146.0	3.9	100.1	29.0	9.3	0.430	7.37	48	50
	+57	146.5	3.8	101.2	25.5	9.4	0.430	7.44	37	49
4	−9	141.5	3.9	96.5	28.2	9.8	0.480	7.44	41	51
(21)	+14	143.0	4.0	96.5	28.7	9.9	0.500	7.34	51	49
	+48	140.5	3.9	97.5	25.7	9.8	0.480	7.48	34	51
5	−10	140.3	4.2	100.7	26.5	9.4	0.455	7.44	38	50
(30)	+18	142.5	4.3	101.5	27.0	9.7	0.472	7.33	52	48
	+55	140.3	3.9	101.5	24.3	9.4	0.455	7.44	37	49
6	−45	141.0	3.8	102.3	26.3	8.0	0.400	7.28	53	45
	− 9	143.0	3.8	102.6	26.5	7.9	0.400	7.33	48	46
(22)	+12	142.4	4.1	100.9	29.2	8.4	0.430	7.26	62	47
	+31	142.0	3.9	100.8	26.7	8.3	0.420	7.34	48	47
	+90	143.0	3.8	100.6	27.0	8.1	0.410	7.37	45	48

* In Experiments 2 to 5, inclusive, arterialized cutaneous, rather than direct arterial, blood was obtained and the plasma content of CO_2 calculated from that of whole blood. Venous serum was used for analysis of Na, K and Cl in Experiments 3, 4, and 5.

† Time as indicated is measured from start of CO_2 inhalation; values obtained during CO_2 inhalation are separated by horizontal single lines.

‡ pH was determined on whole blood but is a measure of the hydrogen ion concentration of plasma; calculated P_{CO_2} is identical for both plasma and whole blood.

§ $(BB)_b$ indicates whole blood buffer base concentration.

interstitial fluid, and the calculated volumes of the several fluid phases, all of which are necessary to the calculation of the ionic transfers shown in Tables III A and III B.

Extracellular bicarbonate and buffer base, and intracellular hydrogen (Tables III A and III B, Figures 3 and 4). In respiratory alkalosis, at the end of the stimulus, the total extracellular bicarbonate changed by a mean of − 136 mEq. with a reciprocal change in total non-bicarbonate buffer anion of + 39 mEq. In respiratory acidosis the same changes were + 32 mEq. and − 12 mEq., respectively. The algebraic sum of these two values, the change in total extracellular buffer base, was − 97 mEq. in respiratory alkalosis and + 20 mEq. in respiratory acidosis. All values except the last were significant at the 5 per cent level. However, from the end of the CO_2 inhalation to the end of the recovery period a change in the opposite direction occurred with the loss of 36 mEq. of extracellular buffer base. This recovery change was highly significant ($p < 0.001$), a finding adding strong support to the contention that the initial response during CO_2 inhalation was real. When these values are corrected for the small amounts of buffer base excreted with bicarbonate and conserved by the secretion of acid and ammonium by the kidney, they provide a quantitative estimate of the transfer between the extracellular and intracellular phases of hydrogen ion in one direction or bicarbonate in the other (Figure 4). Thus, taken as change in the former, the mean change in *intracellular hydrogen* was − 92 mEq. in respiratory alkalosis and + 22 mEq. in respiratory acidosis. Once again, while the mean change in intracellular hydrogen between control periods and the end of the stimulus was significant in the alkalosis group only ($p = 0.005$), the mean reciprocal change from the end of the stimulus to

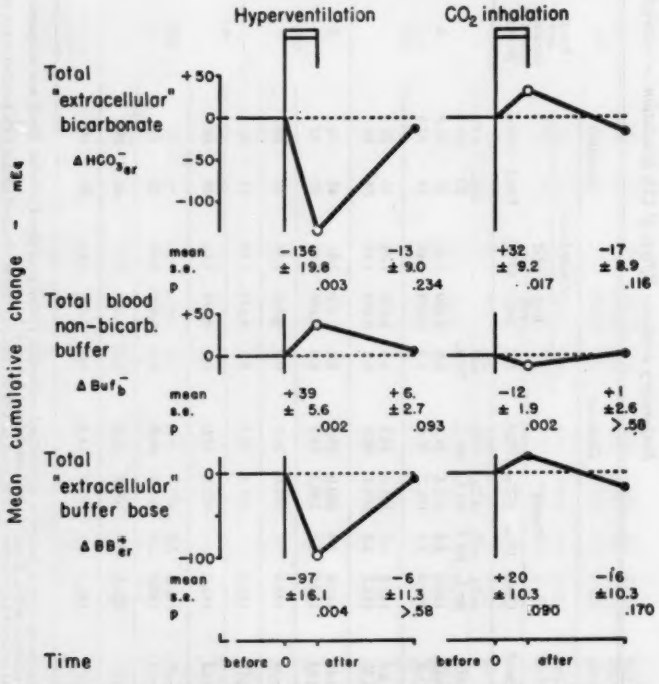

FIG. 3. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN TOTAL "EXTRACELLULAR BICARBONATE," TOTAL BLOOD NON-BICARBONATE BUFFER ANION, AND TOTAL "EXTRACELLULAR" BUFFER BASE

The data are presented as in Figure 1.

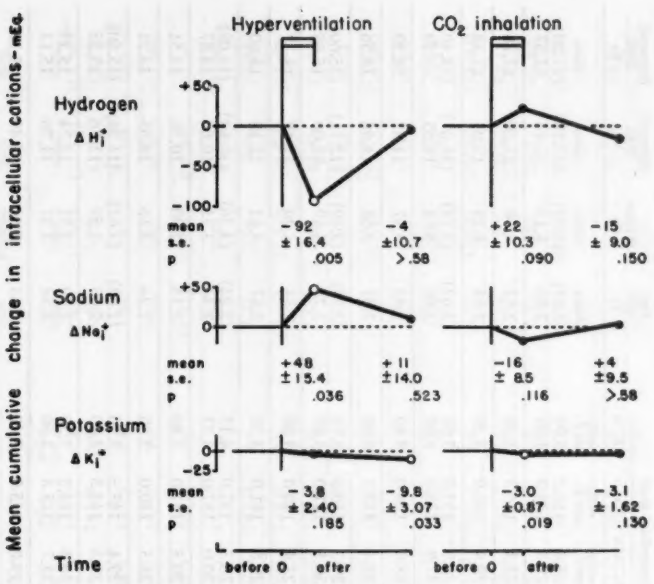

FIG. 4. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS: MEAN CHANGES IN INTRACELLULAR HYDROGEN, SODIUM, AND POTASSIUM

The data are presented as in Figure 1.

the end of recovery following CO_2 inhalation was highly significant (-37 mEq., $p < 0.001$).

Intracellular sodium and potassium (*Tables III A and III B, Figure 4*). In respiratory alkalosis *sodium* decreased in the chloride space to a far greater extent than it was lost in the urine; in respiratory acidosis sodium increased or was unchanged in this phase. On the assumption that the chloride space is essentially equivalent to the extracellular fluid, these data indicate that sodium moved into the intracellular phase by a mean value of $+48$ mEq. during respiratory alkalosis; during respiratory acidosis the mean change of -16 mEq. was in the opposite direction but was not statistically significant. During the recovery period in each type of disturbance the intracellular sodium returned to the control levels; the change from the end of the CO_2 inhalation to the end of the recovery period being $+20$ mEq., $p = 0.04$.

Intracellular *potassium* exhibited changes that were smaller in magnitude, the mean changes being -3.8 and -3.0 mEq. during respiratory alkalosis and acidosis, respectively. Only the latter value was significant, although a significant decrease in intracellular potassium was found during the *recovery* period in respiratory alkalosis.

The quantity $\Delta H_i \sim \Delta Na_i$ represents the sum of the transfer of hydrogen ion in one direction and transfer of sodium in the opposite direction. Since potassium transfers were small, $\Delta H_i \sim \Delta Na_i$ in these experiments was essentially the same as the total cation exchange. The value for this change in the case of respiratory alkalosis was 140 mEq. from the control state to the end of hyperventilation, and 123 mEq. between the end of the stimulus and the end of recovery. In the case of respiratory acidosis the changes in this quantity were 37 and 56 mEq., respectively. All four of these figures are highly significant ($p < 0.001$) and indicate that the calculated exchange of hydrogen for sodium across the phase boundary under conditions of these experiments was not a random phenomenon.

Interrelationships of cation transfers are illustrated in Figures 5 A and 5 B, in which the pattern of response in each group of experiments is shown. In respiratory alkalosis (Figure 5 A), the change in total extracellular bicarbonate (de-

TABLE II A

Respiratory alkalosis: External balances and concentrations of ions in blood and interstitial fluid; and calculated volumes of certain phases of the body fluids †

Expt.	Time†	Balance of				Concentration in						Volume of			
						Whole blood		Interstitial fluid				Red cells V_r	Plasma V_p	Interstit. fluid V_i	Extracell. fluid V_e
		Cl^-	H^{+*}	Na^+	K^+	$(Cl^-)_b$	$(HCO_3^-)_b$	$(Cl^-)_i$	$(HCO_3^-)_i$	$(Na^+)_i$	$(K^+)_i$				
	min.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*liters*	*liters*	*liters*	*liters*
3	−53					85.2	20.5	114.5	28.4	135.2	3.90	(2.87)	(4.01)	(13.19)	(17.20)
	0	−9	−1	−12	−7.0	77.8	21.6	115.0	29.3	138.0	4.00	2.84	4.12	13.45	17.57
	+30	−8	+4	−14	−7.4	78.0	15.5	116.7	22.4	134.5	3.40	2.82	3.98	13.30	17.28
	+121	−5	−3	−13	−5.9	82.0	21.5	114.0	29.4	136.0	3.20	2.84	4.29	13.09	17.38
4	−74					79.0	18.8	116.0	26.9	135.0	3.60	(3.62)	(3.73)	(14.67)	(18.40)
	0	−8	−5	−8	−5.5	77.5	19.5	115.5	27.9	138.2	3.60	3.66	3.84	14.65	18.49
	+36	−7	+3	−7	−8.5	76.0	12.2	117.0	18.9	134.0	3.50	3.43	3.57	14.82	18.39
	+116	−4	−2	−3	−5.7	(79.6)	19.5	113.8	28.4	135.5	3.50	3.57	3.68	14.88	18.56
5	−48					83.8	21.0	117.1	29.3	140.0	3.70	(2.75)	(3.49)	(12.11)	(15.60)
	0	−9	−3	−12	−4.8	83.8	22.0	117.8	30.5	141.0	3.80	2.75	3.49	12.00	15.09
	+32	−10	+4	−14	−6.1	83.2	15.9	118.2	23.4	140.0	3.80	2.63	3.20	12.17	15.37
	+117	−8	−1	−11	−9.4	84.0	21.3	115.2	29.8	141.0	3.80	2.67	3.42	12.18	15.60
6	−49					79.0	20.8	108.5	28.3	137.0	4.15	(2.24)	(3.36)	(10.64)	(14.00)
	0	−28	−3	−25	−7.5	81.0	21.7	105.3	30.0	137.0	4.25	2.30	3.25	10.64	13.87
	+31	−17	+6	−17	−9.9	75.2	14.1	110.8	20.5	137.0	3.80	2.15	2.90	10.58	13.51
	+116	−22	−2	−20	−6.9	79.4	20.3	109.8	28.3	140.0	3.80	2.24	3.16	10.05	13.21
7	−54					80.2	—	111.3	29.4	145.5	3.70	(2.62)	(3.62)	(11.98)	(15.60)
	0	−14	−2	−14	−6.4	80.2	21.2	112.0	29.3	145.5	3.60	2.65	3.59	11.76	15.35
	+28	−8	+2	−8	−5.3	79.7	18.5	112.8	26.3	144.5	3.50	2.59	3.51	11.74	15.25
	+59	−8	+4	−7	−6.9	80.0	17.9	112.8	25.7	145.5	3.50	2.58	3.37	11.76	15.13
	+145	−7	−2	−8	−5.1	80.2	21.0	112.4	29.0	145.0	3.40	2.65	3.66	11.49	15.15

* The balance of hydrogen is non-respiratory; since intake was zero, it is taken to be equivalent with a negative sign to the renal excretion of hydrogen ion, as calculated in Equation *10*.

† Data are expressed per individual periods which end at the time indicated. Time indicated is measured from start of hyperventilation or CO_2 inhalation; values obtained during the stimulus are separated by horizontal single lines; symbols at heads of columns are defined in text; values in parentheses are derived as explained in the text.

TABLE II B

Respiratory acidosis: External balances and concentrations of ions in blood and interstitial fluid; and calculated volumes of certain phases of the body fluids †

Expt.	Time†	Balance of				Concentration in						Volume of			
						Whole blood		Interstitial fluid				Red cells V_r	Plasma V_p	Interstit. fluid V_i	Extracell. fluid V_e
		Cl^-	H^+*	Na^+	K^+	$(Cl^-)_b$	$(HCO_3^-)_b$	$(Cl^-)_i$	$(HCO_3^-)_i$	$(Na^+)_i$	$(K^+)_i$				
	min.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*liters*	*liters*	*liters*	*liters*
1	−37					78.2	23.6	110.7	32.2	140.0	3.4	(2.42)	(3.65)	(11.55)	(15.20)
	+4	−10	+2	−12	−3.2	77.4	23.2	109.9	31.5	141.0	3.4	2.43	3.72	11.51	15.23
	+33	−12	+1	−16	−1.3	78.2	25.3	109.2	34.6	145.5	3.4	2.49	3.43	11.60	15.12
	+97	−18	−1	−26	−1.6	78.4	22.7	108.7	31.4	142.0	3.3	2.46	3.61	11.39	15.00
2	−2					(78.0)	22.6	109.1	32.3	147.0	—	(2.32)	(2.96)	(10.24)	(13.20)
	+30	−4	+3	−7	−3.6	80.0	23.3	109.6	33.5	148.0	—	2.29	2.99	10.10	13.09
	+67	−5	+3	−8	−2.8	79.3	21.3	110.7	30.6	147.0	—	2.32	2.96	9.96	12.95
3	−1					(78.0)	20.5	109.3	29.8	148.0	3.8	(2.59)	(3.57)	(11.83)	(15.40)
	+29	−6	+3	−8	−2.2	77.9	22.3	108.1	36.9	148.0	3.8	2.65	3.51	11.89	15.40
	+63	−9	+3	−12	−2.7	77.5	19.8	109.3	28.1	148.5	3.7	2.67	3.55	11.66	15.31
4	− 2					(76.0)	21.4	104.2	30.9	143.5	3.8	(2.69)	(2.91)	(11.09)	(14.00)
	+22	−4	0	−5	−2.0	78.0	21.6	104.2	31.3	145.0	3.9	2.67	2.87	11.00	13.85
	+53	−5	+1	−7	−1.6	76.0	19.6	105.3	28.7	142.5	3.8	2.69	2.91	10.88	13.79
5	0					(78.0)	20.1	108.8	28.9	142.3	4.1	(2.55)	(3.05)	(10.95)	(14.00)
	+30	−3	+1	−5	−2.6	79.2	21.8	109.6	31.3	144.5	4.2	2.54	2.83	10.94	13.75
	+61	−5	0	−6	−2.4	78.5	19.4	109.6	28.1	142.3	3.8	2.55	3.05	10.77	13.83
6	−51					80.6	21.6	110.5	29.0	143.2	3.7	(2.36)	(3.56)	(11.24)	(14.80)
	0	−3	−2	−3	−5.6	81.3	21.0	110.8	28.4	145.2	3.7	2.40	3.60	11.08	14.68
	+26	−3	0	−4	−2.3	79.5	23.4	109.0	31.6	144.6	3.9	2.43	3.22	11.60	14.82
	+96	−11	−3	−12	−8.0	81.6	21.7	108.7	29.6	145.2	3.6	2.40	3.45	11.27	14.72

* The balance of hydrogen is non-respiratory; since intake was zero, it is taken to be equivalent with a negative sign to the renal excretion of hydrogen ion, as calculated in Equation *10*.

† Data are expressed per individual periods which end at the time indicated. Time indicated is measured from start of hyperventilation or CO_2 inhalation; values obtained during the stimulus are separated by horizontal single lines; symbols at heads of columns are defined in text; values in parentheses are derived as explained in the text.

TABLE III A

Respiratory alkalosis: Calculated changes in ionic contents of the several phases of the body fluids †

Expt.	Time	Red cells		Plasma				Interstitial fluid				Whole blood	Extracellular fluid				Intracellular fluid		
		ΔCl_c^-	ΔHCO_{3c}^-	ΔCl_p^-	ΔHCO_{3p}^-	ΔNa_p^+	ΔK_p^+	ΔCl_i^-	ΔHCO_{3i}^-	ΔNa_i^+	ΔK_i^+	ΔBuf_b^-	ΔHCO_{3ec}^-*	ΔBB_{ec}**	ΔNa_e^+	ΔK_e^+	ΔH_i^+	ΔNa_i^+	ΔK_i^+
	min.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*
3	−53																		
	0	−59	+3	+14	+6	+26	+0.9	+36	+19	+72	+2.5	−15	+28	+13	+98	+3.4	+12	−110	−10.4
	+30	−3	−17	−9	−28	−33	−2.9	+4	−96	−68	−9.0	+46	−141	−95	−101	−11.9	−91	+87	+4.5
	+121	+31	+16	+23	+33	+47	+0.2	−59	+87	−9	−3.0	−36	+136	+100	+38	−2.8	+97	−51	−3.1
4	−74																		
	0	−9	+3	+10	+5	+26	+0.4	−9	+14	+45	−0.1	+4	+22	+26	+71	+0.3	+21	−79	−5.8
	+36	−26	−27	−24	−34	−51	−1.1	+43	−128	−37	−0.8	+56	−189	−133	−88	−1.9	−130	+81	−6.6
	+116	(+37)	+25	+1	+32	+21	+0.2	−42	+142	+27	+0.3	−43	+199	+156	+48	+0.5	+154	−51	−6.2
5	−48																		
	0	−1	+2	+1	+4	+1	+0.3	−9	+11	−3	+0.5	−3	+17	+14	−2	+0.8	+11	−10	−5.6
	+32	−9	−17	−29	−27	−41	−1.1	+28	−81	+11	+0.5	+37	−125	−88	−30	−0.6	−84	+16	−5.5
	+117	+13	+13	+14	+23	+33	+0.8	−35	+78	+15	+0.5	−29	+114	+85	+48	+1.3	+84	−59	−10.7
6	−49																		
	0	+26	+1	−21	+2	−14	−0.2	−33	+18	0	+1.0	+11	+21	+32	−14	+0.8	+29	−11	−8.3
	+31	−50	−16	−18	−33	−47	−2.9	+51	−103	−10	−5.0	+28	−152	−124	−57	−7.9	−118	+40	−2.0
	+116	+24	+13	+24	+26	+44	+1.0	−70	+68	−43	−2.0	−29	+107	+78	+1	−1.0	+76	−21	−5.9
7	−54																		
	0	+1	—	−1	−1	−5	−0.4	−14	−8	−30	−2.0	—	—	—	−35	−2.4	—	+21	−4.0
	+28	−8	−8	−6	−11	−15	−0.7	+6	−35	−13	−1.5	+18	−54	−36	−28	−2.2	−34	+20	−3.1
	+59	+4	−1	−14	−5	−16	−0.5	+2	−7	+13	0	+8	−13	−5	−3	−0.5	−1	−4	−6.4
	+145	0	+9	+27	+17	+41	+0.7	−36	+31	−46	−2.0	−25	+55	+30	−5	−1.3	+28	−3	−3.8

* These values include changes in red cells as well as those in the "extracellular fluid" or chloride space.

† Data are expressed per individual periods which end at the time indicated; time as indicated is measured from start of hyperventilation or CO_2 inhalation; values obtained during the stimulus are separated by horizontal single lines; symbols at heads of columns are defined in text.

TABLE III B

Respiratory acidosis: Calculated changes in ionic contents of the several phases of the body fluids †

Expt.	Time	Red cells		Plasma				Interstitial fluid				Whole blood	Extracellular fluid				Intracellular fluid		
		ΔCl_c^-	ΔHCO_{3c}^-	ΔCl_p^-	ΔHCO_{3p}^-	ΔNa_p^+	ΔK_p^+	ΔCl_i^-	ΔHCO_{3i}^-	ΔNa_i^+	ΔK_i^+	ΔBuf_b^-	ΔHCO_{3ec}^-*	ΔBB_{ec}^{+}*	ΔNa_e^+	ΔK_e^+	ΔH_i^+	ΔNa_i^+	ΔK_i^+
	min.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*
1	−37																		
	+4	−2	+1	+4	−1	+13	+0.2	−12	−10	+4	−0.2	+1	−10	−9	+17	0	−7	−29	−3.2
	+33	+16	+6	−29	+1	−25	−1.0	+1	+39	+66	+0.3	−7	+46	+39	+41	−0.7	+40	−57	−0.6
	+97	−3	−8	+15	−4	+13	+0.3	−30	−44	−73	−1.9	+14	−56	−42	−60	−1.6	−43	+34	0
2	−2																		
	+30	+6	0	+4	+4	+7	—	−14	+8	−10	—	−8	+12	+4	−3	—	+7	−4	—
	+67	−3	−2	0	−9	−7	—	−2	−33	−30	—	+4	−44	−40	−37	—	−37	+29	—
3	−1																		
	+29	+10	+7	−9	+4	−9	−0.2	−7	+27	+9	+0.3	−19	+38	+19	0	+0.1	+22	−8	−2.3
	+63	−6	−6	+7	−8	+8	−0.2	−10	−51	−27	−2.0	+10	−65	−55	−19	−2.2	−52	+7	−0.5
4	−2																		
	+22	+10	+1	−4	−1	−1	+0.1	−10	+2	+3	+0.6	−14	+2	−12	+2	+0.7	−12	−7	−2.7
	+53	−13	−5	+7	−5	−1	−0.1	+1	−31	−44	−1.5	+20	−41	−21	−45	−1.6	−20	+38	0
5	0																		
	+30	+9	+6	−20	−1	−25	−0.6	+8	+26	+22	+1.1	−15	+31	+16	−3	+0.5	+17	−2	−3.1
	+61	−9	−6	+22	−2	+25	−0.3	−18	−40	−49	−5.0	+15	−48	−33	−24	−5.3	−33	+18	+2.9
6	−51																		
	0	+6	−1	+5	−1	+13	+0.2	−14	−12	0	−0.6	+6	−14	−8	+13	−0.4	−10	−16	−5.2
	+26	+5	+7	−44	−1	−56	−0.5	+36	+44	+67	+4.2	−9	+64	+55	+11	+3.7	+55	−15	−6.0
	+96	+6	−6	+22	+1	+35	−0.1	−39	−35	−40	−4.6	+14	−42	−28	−5	−4.7	−31	−7	−3.3

* These values include changes in red cells as well as those in the "extracellular fluid" or chloride space.

† Data are expressed per individual periods which end at the time indicated; time as indicated is measured from start of hyperventilation or CO_2 inhalation; values obtained during the stimulus are separated by horizontal single lines; symbols at heads of columns are defined in text.

picted on the left in each figure) was mostly due to extra-renal, *i.e.*, respiratory, loss from the body; a relatively small portion was excreted by the kidney in association with the fixed cations, potassium and sodium. On the right in each figure are shown the concomitant transfers of cations. The large respiratory losses of bicarbonate as carbonic acid required hydrogen ion which came in part from the protein buffers of the blood and in part from the body cells. The decrement of cellular hydrogen was replaced in part by an increment of cellular sodium; some portion of the remainder was associated with a transfer of undetermined anion (see below). Changes in cellular potassium were relatively insignificant.

In respiratory acidosis (Figure 5 B), the opposite pattern of electrolyte transfers obtained, though to a somewhat lesser degree. The increase in total extracellular bicarbonate resulted from the CO_2 inhalation rather than changes in renal excretion. The associated hydrogen ion was taken up by the protein buffer anions of the blood and by body cells. Simultaneously cellular sodium diminished while cellular potassium was essentially unchanged.

Thus our derived results indicate that acute re-

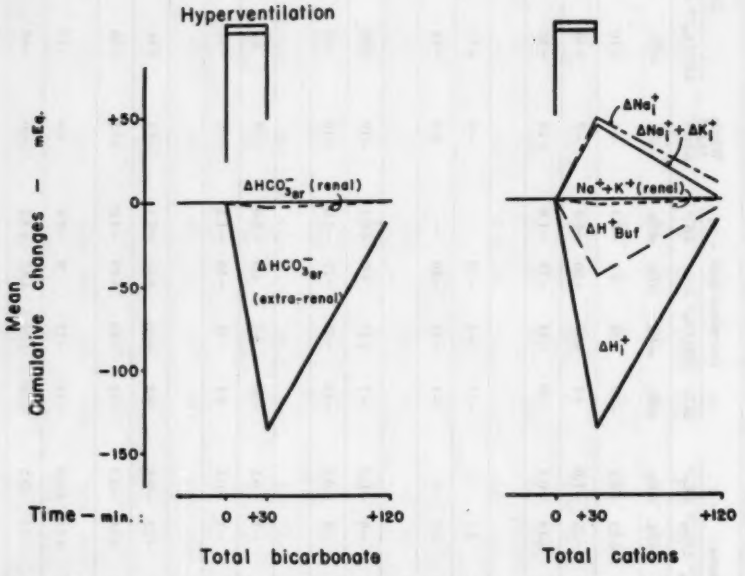

FIG. 5A. RESPIRATORY ALKALOSIS: THE MEAN SUMMATED PATTERNS OF RESPONSE OF TOTAL EXTRACELLULAR BICARBONATE AND OF TOTAL BODY CATIONS, TO HYPERVENTILATION

Changes are plotted cumulatively from the onset to the end of the stimulus and to the end of the recovery period. On the left the dotted line indicates change in bicarbonate by renal excretion; the solid line indicates the total change in extracellular bicarbonate, the difference being extra-renal or respiratory. On the right are depicted the corresponding changes in cations. The dotted line represents renal excretion of sodium and potassium, the dashed line respiratory loss of hydrogen from blood buffer proteins, and the heavy line total hydrogen loss. The difference between the two latter represents transfer of hydrogen from total body cells. The associated changes in intracellular sodium plus potassium and in intracellular sodium alone are indicated above by the solid line and dashed and dotted line, respectively.

Loss of extracellular bicarbonate by the respiratory route greatly exceeds that by the renal. Associated with the loss of bicarbonate through the lungs is the hydrogen ion drawn in part from the protein buffers of the blood and in part from body cells. The latter decrement is partially replaced by transfer of sodium into cells.

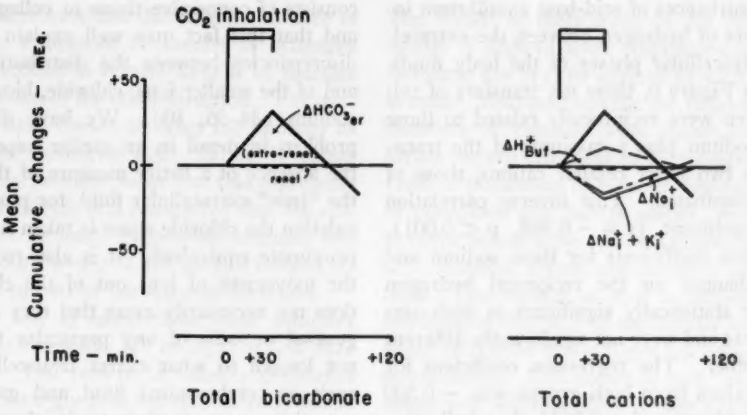

FIG. 5B. RESPIRATORY ACIDOSIS: THE MEAN SUMMATED PATTERNS OF RESPONSE OF TOTAL EXTRACELLULAR BICARBONATE AND OF TOTAL BODY CATIONS, TO CO_2 INHALATION

Data are presented as in Figure 5A.

Extracellular bicarbonate is increased in respiratory retention, and hydrogen is taken up by blood protein buffers and by body cells. Simultaneously the body cells lose sodium.

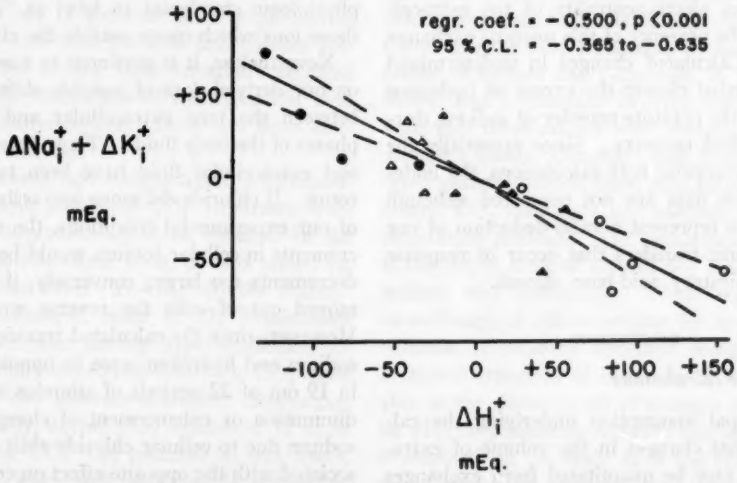

FIG. 6. RESPIRATORY ALKALOSIS AND ACIDOSIS: RELATION OF CALCULATED TRANSFERS OF CELLULAR HYDROGEN TO THOSE OF CELLULAR SODIUM PLUS POTASSIUM

Solid circles (representing alkalosis) and triangles (representing acidosis) indicate changes during the period of stimulus; open circles and triangles indicate changes from the end of the stimulus to the latter part of the recovery period.

The inverse correlation between the two variables indicates a reciprocal relationship between transfers of these cellular cations. The relationship, as indicated by the regression coefficient (solid line), is approximately that of two hydrogen ions in exchange for one sodium or potassium ion.

spiratory disturbances of acid-base equilibrium involve transfers of hydrogen between the extracellular and intracellular phases of the body fluids. As shown in Figure 6, these net transfers of cellular hydrogen were reciprocally related to those of cellular sodium plus potassium; of the transfers of these two latter cellular cations, those of sodium predominated. This inverse correlation is highly significant ($r = -0.868$, $p < 0.001$). The regression coefficients for these sodium and potassium changes on the reciprocal hydrogen change were statistically significant in both sets of experiments and were not significantly different from each other. The regression coefficient for the pooled values from both groups was -0.500 with a standard error of ± 0.0634, thus indicating a 95 per cent chance that the true value lies between -0.365 and -0.635. The magnitude of this relationship therefore suggests a two-for-one exchange, *i.e.*, under these particular conditions of either acute respiratory acidosis or alkalosis approximately two hydrogen ions move for one ion of sodium and potassium considered together. Transfers of undetermined anion may explain the maintenance of electroneutrality of the extracellular fluid in the presence of this unequal exchange of cations. Calculated changes in undetermined ion approximated closely the excess of hydrogen transfer over the opposite transfer of sodium, during stimulus and recovery. Since essentially the same factors comprise both calculations, the undetermined anion data are not presented although they appear to represent a valid deduction of one link in the ionic transfers that occur in response to these respiratory acid-base stimuli.

DISCUSSION

Validity of the calculations

The principal assumption underlying the calculations is that changes in the volume of extracellular fluid may be quantitated from exchanges of chloride, *i.e.*, the chloride space is equated with the true extracellular volume. We recognize that chloride is present in some cells and that, strictly speaking, the chloride space cannot be equated with the "true" extracellular space. However, there is an increasing body of evidence to indicate that larger molecules, such as inulin, fail to penetrate that portion of the extracellular fluid which consists of connective tissue or collagen (32, 33), and that this fact may well explain some of the discrepancies between the distribution of inulin and of the smaller ions, chloride, bicarbonate, and sodium (34–36, 10). We have discussed this problem in detail in an earlier paper (10). In the absence of a better measure of the volume of the "true" extracellular fluid, for purposes of calculation the chloride space is taken here as its approximate equivalent. It is also recognized that the movement of ions out of the chloride space does not necessarily mean that they enter cells in general or cells of any particular tissue. It is not known to what extent transcellular "pools" such as cerebrospinal fluid and gastrointestinal secretions may participate in these exchanges. Sodium has been shown to be readily mobilized from bone in certain animals (37, 38). But the fact that the cells of the bulk tissues, such as skeletal muscle, contain little chloride and much protein (a potential buffer), and the fact that muscle analyses by various workers have indicated changes in ionic concentration in response to acid-base disturbances, make it reasonable from the physiologic standpoint to label as "intracellular" those ions which move outside the chloride space.

Nevertheless, it is pertinent to assess the effect on our derived data of possible shifts of chloride between the true extracellular and intracellular phases of the body fluids. Those between red cells and extracellular fluid have been taken into account. If chloride did move into cells under either of our experimental conditions, the calculated increments in cellular sodium would be too small or decrements too large; conversely, if chloride had moved out of cells the reverse would be true. However, since the calculated transfers of cellular sodium and hydrogen were in opposite directions in 19 out of 22 periods of stimulus and recovery, diminution or enhancement of change in cellular sodium due to cellular chloride shift would be associated with the opposite effect on cellular hydrogen. Therefore the occurrence of such shifts of chloride, unallowed-for in the calculations, would alter the quantitative reciprocal relationship between changes in cellular sodium and hydrogen, but would not invalidate the evidence that transfers of one or both cations took place across the phase boundary.

The calculated difference between change in ex-

tracellular buffer base and the external balance of bicarbonate is a measure of transfer of hydrogen ion in one direction in respect to cells or transfer of bicarbonate ion in the other direction. For convenience we have chosen to label the findings as movements of *hydrogen ion*. While it seems somewhat more probable to us that this represents the transfer involved, it is recognized that the distinction cannot be made by present methods and that the net result would be the same if instead bicarbonate ion moved in the opposite direction.

The major role of intracellular ionic transfers in buffering extracellular pH changes in acid-base disturbances. The results of these experiments indicate very strongly that the cells of body tissue provide a major buffer system against the effects of acute acid-base disturbances of the respiratory type. Ninety per cent or more of the change in effective extracellular bicarbonate was extra-renal and was related to changes in hydrogen uptake or release by buffer protein in the blood and by ionic transfer into, or out of, cellular fluid in the body; only a very small fraction was accounted for by acid-base adjustments in the kidney over the short periods of time of these experiments. Exchanges of hydrogen ion with blood buffer systems and in the kidney have heretofore received much attention; exchanges of hydrogen ion with intracellular buffer systems have not been considered so extensively.

Earlier workers had demonstrated experimentally that a major portion of CO_2 withdrawn from the body by hyperventilation, or retained in the body by inhalation, came from, or was added to, tissues other than blood (39–41). On the assumption of an unchanged basal R. Q., Rosenbaum (42) calculated that during hyperventilation 21 to 47 per cent of expired non-metabolic CO_2 came from outside the chloride-corrected thiocyanate space; conversely, during CO_2 inhalation, 12 to 56 per cent of the CO_2 was retained in this space. These studies clearly indicated that the intracellular fluid shared in the buffering of respiratory acid-base disturbances, but there was not sufficient appreciation of the obligatory nature of associated transfers of hydrogen and other cations.

These associated ionic transfers are delineated in our experiments and in the important paper by Giebisch, Berger, and Pitts (9) which has been published since our experiments were completed. The results of their study of the extra-renal response of dogs to acute respiratory acid-base disturbances are in close agreement with our findings in man. Measured against the radio-sulfate space, transfers of chloride were found to occur only across the red cell membrane; transfers of sodium (and of potassium to a lesser degree), as well as of phosphate and lactate anions, were calculated to occur across some unkown extracellular boundary. Cellular anions are involved as well as cations. Our results, at least in respiratory alkalosis, indicate that part of the cellular buffering consisted in release of hydrogen ion with an undetermined anion. The nature of this "undetermined" anion was not identified in our experiments but it may well be lactate since an increase in extracellular lactic acid has been specifically reported to occur under these circumstances (43–45).

The relatively few analyses of tissues which are available in respiratory acid-base disturbances are in general agreement with the indirect evidence of our balance experiments. Darrow and Sarason (46) found a high muscle content of intracellular sodium in rats subjected to low oxygen tension and chronic hyperventilation. Malorny (47) found during CO_2 inhalation by animals an increase of sodium and potassium, in whole muscle tissue, and a decrease in liver tissue, and suggests that these cations were exchanging with hydrogen. Cooke, Coughlin, and Segar (48) analyzed skeletal muscle from rats subjected to prolonged CO_2 inhalation and partially compensated respiratory acidosis. The potassium content was at the upper limit of normal and the intracellular sodium was diminished. These results suggest an exchange of cellular sodium for hydrogen.

Acute respiratory acid-base disturbances lead to linked transfers of intracellular cations, probably as the direct result of changes in extracellular pH; no positive or negative load of fixed cation is imposed on the body as is the case in the so-called metabolic disturbances. Intracellular exchanges of hydrogen, sodium, and potassium ions have been more widely recognized in the latter, namely, primary metabolic disturbances of acid-base equilibrium (10–14), as well as in primary deficiency of potassium (1–3, 49–51).

The relation of cellular to renal transfers. The renal and cellular adjustments of respiratory acid-base disturbances are linked reactions. The pre-

cise patterns of these linked reactions depend upon a variety of factors, the chief of which are the intensity and duration of the primary stimulus, the time of observation, and the intrinsic adequacy of renal function. In the experiments reported here the respiratory stimuli and periods of observation were of such short duration that the renal response, although definitive in character, was of very small magnitude in relation to the calculated ionic exchanges between cells and extracellular fluid. Chronic respiratory stimuli may produce a different effect; Sullivan and Dorman (52) have demonstrated that bicarbonate reabsorption by the renal tubule is progressively enhanced in chronic, as compared to acute, respiratory acidosis. And certainly, in clinical examples of chronic respiratory acidosis, the secondary adjustment of the buffer anion concentration in extracellular fluid by the renal excretion of chloride relative to bicarbonate conditions strongly the degree of deviation of hydrogen ion concentration.

The observations reported in this paper together with those of many other investigators, as discussed above, are leading to an enlarged and more precise concept of acid-base regulation. The first and immediate defense of the neutrality of body fluids against an external agent is the *physicochemical mechanism* involved in the action of the buffer systems in blood. Other buffer systems called into play are, next, the bicarbonate-carbonic acid system of the rest of the extracellular fluid (including the collagen subphase), and finally, the organic phosphates and proteins of tissue cells, and some of the bone salts. In addition to the chemical action of these buffers, *physiological mechanisms* aid in neutrality regulation. These include regulation of respiratory minute volume so as to achieve, where possible, a higher, lower, or more normal level of CO_2 pressure in body fluids; changes in cellular metabolism, such as an increase in the rate of formation of organic acids; and renal function, whereby compensatory changes in the ionic structure of plasma are effected. Although all these mechanisms are brought into play almost immediately after the acid-base disturbance is first noted in arterial blood, the full effects of respiratory compensation are achieved very rapidly, whereas those due to metabolic or renal compensation reach their maximum much later. And although all these mechanisms serve to protect the relative constancy of pH in body fluids, the kidney is primarily responsible for the ultimate restoration of a normal ionic pattern.

SUMMARY

Ionic tranfers between the extracellular fluid (assumed to be 20 per cent of body weight with changes estimated on the basis of the chloride space) and the "intracellular" fluid were calculated in five normal subjects during and after acute respiratory alkalosis produced by hyperventilation; in another group of six subjects the same transfers were calculated during and after acute respiratory acidosis produced by CO_2 inhalation. Direct calculations were made of sodium and potassium transfers as well as indirect calculations of transfers of intracellular hydrogen on the basis of changes in bicarbonate and other buffer anions in the extracellular fluid and red cells. The results were as follows:

1. In respiratory alkalosis, hydrogen left cells and sodium entered cells.

2. In respiratory acidosis hydrogen tended to enter, and sodium to leave, cells, but these mean changes were not quite significant at the 5 per cent level.

3. In both types of experiments the sums of hydrogen transfer in one direction and of sodium in the other were highly significant. The ratio of hydrogen transfer to sodium transfer in the opposite direction was approximately 2 to 1 under these conditions; a 1 to 1 transfer of anion with that of hydrogen not exchanged for sodium appeared to be involved.

4. In both types of experiments the changes in cell potassium were much smaller in magnitude or were negligible, for the short duration of the stimuli.

It is concluded that in acute respiratory acid-base disturbances a large part of the immediate buffering of the extracellular fluid is achieved by exchanges of hydrogen for sodium across the cell boundaries in body tissues.

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THE EFFECT OF POTASSIUM ON INTRACELLULAR BICARBONATE IN SLICES OF KIDNEY CORTEX [1]

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An increase in serum bicarbonate concentration has been observed in association with potassium depletion in a variety of clinical and experimental conditions. Although the development of the alkalosis may be extra-renal in origin, its perpetuation must depend upon an alteration in renal function which can be characterized as either an increase in bicarbonate reabsorption or an increase in hydrogen ion excretion. Many instances of so-called "paradoxical" aciduria have been described (*cf.* 1, 2).

Recent attempts to interpret this phenomenon have been predicated upon the concept that the extracellular alkalosis of potassium depletion is accompanied by an intracellular acidosis. Supporting evidence has been derived from a variety of experiments. In the muscle of potassium depleted rats Gardner, MacLachlan, and Berman (3) observed a decrease in the intracellular pH as calculated from whole tissue analyses corrected for extracellular space. Cooke and co-workers (4), on the basis of balance experiments and muscle analyses, concluded that the loss of intracellular potassium in rats was accompanied by an intracellular accumulation of both sodium and hydrogen ions. In studying experimental potassium depletion in man, Black and Milne (5) interpreted their balance data as indicating a shift of hydrogen ions into cells. In an attempt to obtain direct evidence relating the electrolyte composition of the renal parenchyma to the excretion of acid urine, Darrow, Cooke, and Coville (6) analyzed the kidneys of potassium depleted rats, but with inconclusive results.

Since the intracellular sodium and potassium concentrations in the kidney cortex can be varied over a wide range by the use of *in vitro* techniques, studies of tissue slices were undertaken in order to define the relationships of intracellular potassium and bicarbonate.

METHODS

The preparation of tissues has been described previously in detail (7) and is summarized here. Fresh slices were made from the renal cortex of rabbits killed by carotid exsanguination and were depleted of potassium by leaching for one hour in 0.15 N NaCl at room temperature. Half the slices were then incubated in Warburg flasks in 3 ml. of a medium containing $NaHCO_3$ 18 mEq. per L., $CaCl_2$ 1.3 mEq. per L., sodium phosphate buffer (pH 7.4) 3.7 mM. per L. and sodium acetate 0.01 mM. per L.[3] The remaining slices were incubated in a medium identical in composition except for the addition of 10 mEq. per L. of KCl. Sufficient NaCl was added to both media to provide constant osmolar concentrations of 300 mOs. per L. The Warburg vessels were gassed with a mixture of 5 per cent CO_2 and 95 per cent O_2. The carbon dioxide-bicarbonate buffer system provided a pH of 7.4 in the external medium (8). Following incubation for 35 minutes at 25° C. the slices were promptly removed, blotted, and then analyzed for water and electrolyte. This entire procedure will be referred to hereafter as the *standard system;* modification of individual variables will be described for each special circumstance.

Tissue sodium and potassium were determined by flame photometry using lithium as an internal standard. In early experiments tissue chloride was determined by a modified Volhard titration. In later experiments, through the courtesy of Dr. Paul Marks, chlorides were measured potentiometrically using a silver-silver chloride cell (9).

"Acid-labile CO_2" was determined by a modification of the technique devised by Conway and Fearon (10). Conway dishes (inner chamber 40 mm. diameter, 5 mm. high) were prepared with 1.3 ml. of 0.025 N $Ba(OH)_2$ in the center well, covered with ground glass cover-slips and sealed with anhydrous lanolin. After incubation in the Warburg flasks, tissue slices (about 300 mg. per

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[3] Although these amounts of calcium and phosphate were routinely added, their omission did not influence the results.

TABLE I

Effect of potassium on tissue bicarbonate concentration

	Tissue composition		
	HCO_3^-	K^+	$Na^+ + K^+$
	mEq./Kg. wet weight		
Before incubation	—	35.0	137
After incubation			
No K^+ added to external medium	13.5	34.9	138
10 mEq./L. K^+ added to external medium	18.0	77.2	135

vessel) were quickly transferred to the outer well of a prepared Conway dish. About 5 ml. of 2 N H_2SO_4 was then added to the outer well on the side opposite the tissues. At all times the cover glass was slipped open only far enough to allow insertion of the sample or the pipette tip. The dish was promptly resealed and gently rotated to provide complete penetration of acid into the slices. The Conway dishes so loaded with $Ba(OH)_2$, tissue, and H_2SO_4 were allowed to stand at room temperature for one hour. A one ml. aliquot of fluid from the center well was then removed and its content of $Ba(OH)_2$ determined by titration with 0.005 N HCl, using thymol blue as indicator. Calculation by difference revealed the quantity of $Ba(OH)_2$ converted to $BaCO_3$ and hence the amount of "acid-labile CO_2" present in the tissue. A Conway dish without tissue was run with each experiment as a control. Standardization of this technique using known concentrations of $NaHCO_3$ solution in the outer well gave 95 to 105 per cent recoveries. Longer standing, increased temperature, or gentle agitation of the Conway dish did not significantly alter the recovery. In one experiment, through the courtesy of Dr. Duncan Holaday, "acid-labile CO_2" of the slices was measured by the gasometric method of Danielson and Hastings (11).[4] The results were the same as those obtained by the modified Conway technique.

All expressions for the concentration of electrolyte in tissues are given per kilogram wet weight. The qO_2 was calculated as the cubic millimeters of O_2 consumed per hour per mg. initial wet weight of tissue. Tissue bicarbonate determinations were done in duplicate on tissue incubated in separate flasks and treated identically. Tissue Na, K, and Cl were determined in duplicate on the specimen from a single flask.

[4] "Acid-labile CO_2" is considered to include dissolved CO_2, H_2CO_3, and HCO_3^-. For simplicity, except as indicated in Table VIII, the term bicarbonate is used as a synonym for "acid-labile CO_2," since this is by far the largest component of the CO_2–H_2CO_3–HCO_3^- system. It is recognized that Conway and Fearon (10) have presented evidence to suggest that compounds other than CO_2–H_2CO_3–HCO_3^- are measured as "acid-labile CO_2." However, since the nature of these compounds is not clear and confirmation of this observation has not been presented, the conventions of Danielson and Hastings (11) have been adopted here.

RESULTS

A typical experiment employing the *standard system* is illustrated in Table I. Before incubation, the potassium concentration of the leached slice was 35 mEq. per Kg. After incubation in a potassium-free medium there was no change in tissue potassium, but incubation in the medium containing 10 mEq. per L. of potassium resulted in an accumulation of potassium by the tissue to a final concentration of 77.2 mEq. per Kg. This value is similar to that found in fresh tissue (7). The uptake of potassium by the slices was associated with a loss of sodium, so that there was no significant change in the sum of sodium and potassium.[5] The bicarbonate concentration of the tissue incubated without potassium was 13.5 mEq. per Kg.; slices with added potassium had a significantly higher bicarbonate concentration (18.0 mEq. per Kg.).

TABLE II

Average values from 17 experiments employing the Standard System *

	Final tissue composition			
	K^+ added to incubation medium			
	0	10 mEq./L.	Δ ± S.D.	P
HCO_3^-, *mEq./Kg.*	12.3	17.0	4.7± .52	<.001
K^+, *mEq./Kg.*	32.1	71.7	39.6±1.2	<.001
$Na^+ + K^+$, *mEq./Kg.*	140	141	1.0±1.5	>.5
H_2O, *% wet weight*	77.8	78.7	0.9± .24	<.001

* Eight analyses of fresh kidney cortex, removed as quickly as possible after killing the rabbit, yielded an average bicarbonate concentration of 14.4 mEq. per Kg. Fresh tissue values previously reported (7) are: K^+, 69.3 mEq. per Kg.; $Na^+ + K^+$, 138 mEq. per Kg.; H_2O, 77.1 per cent wet weight.

A similar relationship was observed in a total of 72 experiments. In Table II are summarized the results from 17 experiments in which tissue bicarbonate, sodium, potassium and water content were measured. The average difference in tissue bicarbonate between the slices with and without added potassium was 4.7 mEq. per Kg.; this was associated with an average change of 39.6 mEq. per Kg. in the level of tissue potassium.

[5] In this presentation the convention of correlating tissue bicarbonate to potassium will be employed. It is recognized that because of the nature of the cation changes, tissue sodium could be used as a reference equally well.

The sum of tissue sodium and potassium remained constant, although small changes may have escaped detection because of difficulties inherent in the measurement of tissue sodium. The accumulation of potassium was associated with a small, but statistically significant, increase in tissue hydration. As will be noted in subsequent tables, the absolute level of tissue bicarbonate varied somewhat from one experiment to another, but the difference between the slices with low and high tissue potassium remained constant. In every experiment, therefore, suitable internal controls were employed.

The dependence of tissue bicarbonate on the intracellular accumulation of potassium rather than on the potassium concentration in the incubation medium is illustrated by experiments in which respiration or aerobic phosphorylation was inhibited. Previous studies (7) have shown that the uptake of potassium is related to aerobic metabolic activity. The effects of anaerobic incubation

TABLE III

Comparison of aerobic and anaerobic incubation *

Gas	Medium K^+		Tissue	
	Initial	Final	HCO_3^-	K^+
	mEq./L.	*mEq./L.*	*mEq./Kg.*	*mEq./Kg.*
5% CO_2–95% O_2	0	0	14.4	41.3
	10	8.4	17.3	75.3
5% CO_2–95% N_2	0	2.6	10.6	15.3
	10	12.7	10.9	24.5

* Before incubation the tissue contained 42.4 mEq. K^+ per Kg.

are illustrated in Table III. The experimental conditions were the same as those of the *standard system* except that the gas mixture was 5 per cent CO_2–95 per cent N_2 instead of 5 per cent CO_2–95 per cent O_2. The anaerobic tissues showed a net loss of potassium and a tissue bicarbonate concentration unchanged by the addition of potassium to the external medium.

It has been shown that 2,4-dinitrophenol uncouples aerobic phosphorylation and prevents the uptake of potassium by kidney slices (12). As shown in Table IV, this agent causes a failure of bicarbonate accumulation and a parallel inhibition of potassium uptake.

Previous observations have shown that the presence of low concentrations of potassium in the external medium increases the rate of oxygen

TABLE IV

Effect of 2,4-dinitrophenol *

2,4-DNP M	Medium K^+		Tissue composition	
	Initial	Final	HCO_3^-	K^+
	mEq./L.	*mEq./L.*	*mEq./Kg.*	*mEq./Kg.*
0	0	0	13.3	40.2
0	10	6.8	20.5	74.8
3×10^{-5}	10	9.8	16.7	42.0
9×10^{-5}	10	11.6	12.7	28.8
3×10^{-4}	10	11.9	12.7	25.3

* Before incubation the tissue contained 35.9 mEq. K^+ per Kg. The sum of Na^+ plus K^+ remained constant.

consumption (7). This suggested that the change in the tissue level of bicarbonate might be a direct effect of the respiratory stimulation produced by the addition of potassium. The use of the indirect Warburg technique (8) permitted the determination of the qO_2 in the presence of CO_2 in the gas phase. Experiments were carried out with 40 mEq. per L. of potassium in the incubation medium, instead of 10 mEq. per L., since this higher concentration is not generally accompanied by respiratory stimulation. The results are shown in Table V, and clearly indicate that the change in bicarbonate in tissues incubated with added potassium is not dependent upon increased oxygen consumption. In addition, in the earlier experiment with 2,4-dinitrophenol, despite marked respiratory stimulation there was a fall, rather than an increase, in the level of tissue bicarbonate. The substitution of succinate, citrate or α-ketoglutarate for acetate in the *standard system* did not change the tissue level of either bicarbonate or potassium. With succinate as substrate there was approximately a fifty per cent increase in oxygen consumption. It is concluded, therefore, that the effect of potassium on tissue bicarbonate is not the direct result of respiratory stimulation.

The influence of different levels of tissue potassium on bicarbonate was examined over a wide range by varying the amount of potassium chloride added to the medium. The osmolarity was kept constant by adjustments in sodium chloride. The results of four separate experiments are illustrated in Figure 1. It is seen that tissue bicarbonate and potassium are linearly related except at the very high tissue potassium levels, which were achieved by raising the external concentration to as high as 120 mEq. per L. The results of high external concentrations are difficult to evaluate be-

TABLE V

Relation of oxygen consumption to tissue bicarbonate *

Medium K^+	qO_2	Tissue HCO_3^-
mEq./L.		*mEq./Kg.*
0	.63	9.3
40	.54	15.7

* The qO_2 was measured by the indirect method of Warburg (8).

cause of marked respiratory depression and excessive tissue hydration.

The rise in tissue bicarbonate noted above could result from either an increase in the total number of anions or a replacement of some anions by bicarbonate. Evidence supporting the latter mechanism is afforded by two observations: 1) The sum of tissue sodium and potassium remained constant (see Table II); and 2) the changes in tissue chloride which were observed in a series of experiments (Table VI). The average change upon the addition of potassium was + 4.0 mEq. per Kg. of bicarbonate and − 5.7 mEq. per Kg. of chloride. Within the limits of the methods employed, these changes are considered to be approximately equivalent. The substitution of nitrate for chloride in the external medium resulted in a fall in tissue chloride to about 6 mEq. per Kg. The tissue bicarbonates in the nitrate medium were the same as in the chloride medium, and the usual change in tissue bicarbonate was produced by the addition of potassium. The results are interpreted as evidence that the changes in tissue chloride (*cf.* Table VI) associated with the accumulation of potassium are secondary to changes in tissue bicarbonate.

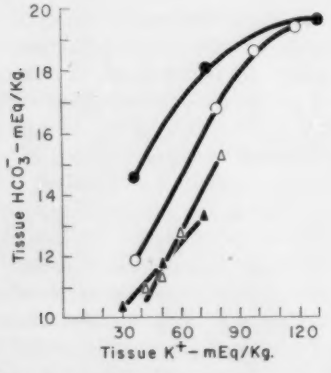

FIG. 1. RELATION OF TISSUE BICARBONATE TO TISSUE POTASSIUM

Lines indicate four separate experiments. Tissue potassium was varied by changing external concentration.

In a number of biological systems it has been reported that rubidium and cesium behave in a manner similar to potassium. As shown in Table VII the addition of the chloride salts of these elements to the incubation medium resulted in changes in tissue bicarbonate resembling those produced by potassium, whereas lithium was ineffective. It is of interest that hypokalemic alkalosis in the rat may be corrected by the administration of rubidium (13). However, the present results with lithium are not what might be anticipated from previous studies in the dog in which it was shown that potassium excretion and urine pH were increased by the administration of this ion (14, 15).

TABLE VI

Relation of tissue chloride and bicarbonate *

HCO_3^-, *mEq./Kg.*			Cl^-, *mEq./Kg.*		
No K^+ added	K^+ added	$\Delta \pm$ S.D.	No K^+ added	K^+ added	$\Delta \pm$ S.D.
15.2	19.2	+4.0±.41	64.9	59.2	−5.7±.52

* Values reported are averages from eight experiments in which the *standard system* was employed. Chloride was determined by a modified Volhard titration in four experiments, and by a potentiometric method (9) in the remainder. Similar results were obtained by both methods. The chloride concentration in the incubation medium was, initially, 118 mEq. per L.

Studies of the effect of changes in the external concentration of bicarbonate were undertaken and the results of a typical experiment are given in Table VIII. In this and five similar experiments, pCO_2 was maintained constant so that the alterations in external bicarbonate were associated with parallel changes in the pH of the medium. The phosphate buffer used in the standard incubation medium was omitted. At low concentrations of external bicarbonate the addition of potassium had a much greater effect on the level of tissue bicarbonate than in the standard system. Conversely, at high external bicarbonate concentrations, the effect of potassium was negligible. The pH of the external solution had no effect on the final tissue concentration of potassium or of potassium plus sodium, either with or without the addition of potassium to the medium. Similarly,

respiration and tissue hydration were essentially the same at each pH.

Because of the changes in external bicarbonate, the data from these experiments can not be evaluated solely in terms of total tissue concentrations. Therefore, intracellular values were calculated on the basis of the assumptions listed in Table VIII. Despite recognized limitations, these data probably represent a reasonable approximation and afford a valid basis for comparison. As shown in Figure 2, in the potassium depleted slice the intracellular concentration of bicarbonate rises as the external level is increased. In contrast, slices with normal tissue potassium maintain nearly constant intracellular bicarbonate despite a five-fold change in external concentration.

TABLE VII

Effect of other cations *

Salt added	Tissue HCO_3^-
	mEq./Kg.
Sodium	14.9
Lithium	15.0
Potassium	19.1
Rubidium	18.4
Cesium	20.5

* Ten mEq. per L. of the chloride salt of the indicated cations was added to the external medium. Total osmolarity was kept constant by the addition of sodium chloride in a concentration of 105 mEq. per L., which, plus the other cup components, provided a total osmolar concentration of 300 mOs. per L. (See Methods.) The ions listed above had no significant effect on the rate of oxygen consumption.

DISCUSSION

These results directly demonstrate that the level of tissue bicarbonate can be influenced by potassium. For reasons previously mentioned these effects may be attributed to intracellular changes. If applied to certain physiological adjustments which are observed in the intact animal, the findings could provide an explanation for the "paradoxical" aciduria of potassium depletion. Recent studies indicate that the exchange of sodium from the tubular urine for hydrogen ion from the tubule cell is the mechanism by which bicarbonate is reabsorbed and the urine acidified (16–18). Berliner, Kennedy, and Orloff (19) have postulated that the sodium-potassium exchange responsible for the tubular secretion of potassium may compete with sodium-hydrogen exchange. For example, the aciduria of potassium depletion might be attributed to a decrease in the rate of potassium secretion and an increase in sodium-hydrogen exchange. The present results may define some of the regulatory factors more precisely, namely that the apparent competition between hydrogen and potassium ions for an excretory mechanism may be a reflection of their relative intracellular concentrations. Thus, the internal environment of the tubule cell may directly modify the composition

TABLE VIII

Effect of external bicarbonate on calculated intracellular pH *

	External HCO_3^-	External pH	Tissue HCO_3^-	"Intracellular" HCO_3^-	"Intracellular" pH
	mEq./L.		*mEq./Kg.*	*mEq./L. ICW*	
No K^+ added	5	6.85	7.1	11	7.00
	18	7.40	13.3	16.5	7.18
	28	7.60	21.0	26.4	7.38
K^+ added 10 mEq./L.	5	6.85	15.8	27.3	7.40
	18	7.40	19.9	29.1	7.42
	28	7.60	22.4	29.1	7.42

* All cups were gassed with 5 per cent CO_2–95 per cent O_2. The pH of the medium was calculated from the nomogram of Umbreit, Burris, and Stauffer (8). The intracellular values were calculated by the method of Wallace and Hastings (21), assuming: 1) tissue solids, 22 per cent of total tissue; 2) extracellular space, 25 per cent of total tissue; 3) pK′ for carbonic acid, 6.1; 4) the same pCO_2 in the intracellular water as in the external medium; 5) solubility coefficient of CO_2 in the water of the intracellular space, 0.592; 6) all acid-labile tissue CO_2 to be bicarbonate ion.

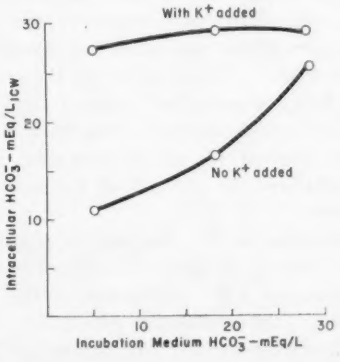

FIG. 2. RELATION OF INTRACELLULAR BICARBONATE TO EXTERNAL BICARBONATE CONCENTRATION

All tissues gassed with 5 per cent CO_2–95 per cent O_2. For initial pH values of media, see Table VIII.

of the urine, and extra-renal regulatory mechanisms need not be implicated.

In their studies on rat skeletal muscle, Cooke, Segar, Cheek, Coville, and Darrow (4) suggested that during the development and repair of hypokalemic alkalosis the quantitative relationships between intra- and extra-cellular univalent cations might be described by an ionic exchange in the ratio of three potassiums for two sodiums and one hydrogen. Because of the difficulties inherent in the estimation of intracellular buffer capacity, the calculation of this ratio is only an approximate description. In the present experiments, at an external pH of 7.4, an exchange of eight sodium for eight potassium ions is accompanied by a simultaneous exchange of one chloride for one bicarbonate ion. Estimates of changes in intracellular hydrogen ion concentration in this system have only limited validity because of the following factors: 1) The exact nature of intracellular "acid-labile" carbon dioxide has not been ascertained (10); and 2) calculations presented above for the kidney cortex have assumed a homogeneous cell population. If the cells do not behave uniformly, the calculated changes would be smaller in some cells and greater in others.

Despite these limitations, the derived intracellular pH suggests that the level of potassium within the cells is related to their buffer capacity. As shown in Table VIII and Figure 2, a normal potassium concentration is associated with only small changes in intracellular pH when the external pH is varied. However, the results do not indicate an absolutely constant relationship between intracellular potassium and hydrogen ion concentrations. This is shown by the results obtained with the potassium depleted slice (Figure 2). Although the level of intracellular potassium remains constant, the pH of these slices is markedly influenced by the pH of the external environment.

While some of the observations reported here are qualitatively similar to those predicted by the Boyle-Conway (20) application of the Donnan equilibrium, the quantitative relationships between intra- and extra-cellular ions deviate so far from the predicted values that the hypothesis need not be considered further. An interpretation of the present results on a physico-chemical basis is not warranted at this time.

SUMMARY

Studies on slices of rabbit kidney cortex have directly demonstrated that the intracellular concentration of bicarbonate is related to that of potassium. These findings support the hypothesis that the pH of the cells of the renal tubules may directly influence renal excretion in the regulation of acid-base balance in conditions of potassium depletion or excess.

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THE INTERACTION OF THYROXINE WITH PLASMA PROTEINS: LOCALIZATION OF THYROXINE-BINDING PROTEIN IN COHN FRACTIONS OF PLASMA [1, 2]

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Recently zone electrophoretic techniques have been employed to characterize the site of thyroxine-binding in plasma (1–7). In most reports, an association has been described between thyroxine and a protein moiety intermediate in electrophoretic mobility at pH 8.6 between the α_1 and the α_2 globulins.

A concentrated source of this thyroxine-binding protein (TBP) has not been available for further purification or for experimental evaluation of its physiological role. To meet these objectives, zone electrophoretic techniques have been employed to localize TBP in subfractions of plasma prepared by Method 6 of Cohn and his co-workers (8, 9).

MATERIALS

1. Cohn Fractions: Fractions were obtained from 250 milliliters of fresh plasma and from 100 liters of plasma derived from out-dated blood-bank blood. Two separate fractionations were performed with ethanol-water mixtures of controlled pH, ionic strength, temperature, and protein concentration. Yields from the large-scale fractionation of old plasma were sufficient to permit characterization of the individual fractions by moving-boundary electrophoresis and by chemical estimation of iodine/nitrogen ratios (Table I).

2. Thyroxine: Chromatographically-pure I^{131}-labelled thyroxine (10), dissolved in propylene glycol was obtained from a commercial source.[4] Gravimetrically determined amounts of unlabelled L-thyroxine sodium [5] were added to vary specific activity.

METHODS

1. Free electrophoresis: [6] Cohn Fractions were dissolved in barbital buffer, pH 8.6, ionic strength 0.1. Two gram per cent solutions were dialyzed against barbital buffer at 5° C. Free moving boundary electrophoresis was performed in a Klett Electrophoresis Apparatus and photographs were obtained with a moving diaphragm scanning method. Pictures were enlarged and analyzed by dropping perpendiculars from the lowest points and measuring the areas under the peaks in the magnified schlieren diagrams.

2. Chemical analyses: Protein-bound iodine (PBI) was determined by a modification of the alkaline ash method of Barker [7] (11). Wet digestion was employed

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[2] Presented in part at the 1955 meeting of the Endocrine Society, June 2–4, 1955, Atlantic City, New Jersey.

[3] Fellow of the American Cancer Society, Inc. recommended by the Committee on Growth of the National Research Council.

[4] Abbott Laboratories, Oak Ridge, Tenn.

[5] This was 96.2 per cent pure on a dry basis and kindly supplied by Dr. A. E. Heming of Smith, Kline & French Laboratories, Philadelphia, Pa.

[6] Analyses performed in the laboratory of Dr. John M. Newell of the Massachusetts Public Health Biologic Laboratories, Jamaica Plain, Mass.

[7] Iodine analyses were performed by Bio-Science Laboratories, Inc., 2231 Carmelina Avenue, Los Angeles 64, Calif.

TABLE I

Moving boundary electrophoresis and iodine analyses of Cohn Fractions

Fraction	Electrophoretic composition (*per cent*)						I^{127}
	Alb.	α_1	α_2	β_1	β_2	γ	$\mu g./g$
I	10*	4*	7*	11*	51*	17*	1.3
II–III	4	—	18	15	21	42	0.9
IV-1	6	—	82	8	4	—	3.3
IV-5	8	61	22	9	—	—	1.3
IV-6	2	18	66	14	—	—	3.7
IV-7	5	—	23	66	6	—	1.0
IV-8	79	11	3	7	—	—	5.4
IV-9	12	—	31	57	—	—	2.6
V	84	14	—	2	—	—	5.8
Supernate V	47	46	7	—	—	—	6.1

* Averages for Fractions I prepared during the past four years at the Massachusetts Public Health Biologic Laboratories. All other analyses were performed with the fractions derived from old plasma which were employed in the present studies.

for measurement of total iodine[7] (12). Protein concentrations were determined by a modified biuret procedure (13); the micro-Kjeldahl technique was used for nitrogen analyses.

3. Thyroxine-protein mixtures: One-tenth ml. of saline containing the desired amounts of labelled and unlabelled thyroxine were added to 0.9 ml. of the various protein solutions. Mixtures of protein and thyroxine were permitted to stand for a minimum of thirty minutes at room temperature to assure binding equilibrium prior to zone electrophoresis. Fresh solutions of stable and radioactive thyroxine were prepared for each experiment. Similarly, freshly-drawn sera and freshly-dissolved Cohn Fractions were routinely employed even though thyroxine-binding was apparently not altered by storage in the deep-freeze. In all experiments, the final absolute concentration of organic iodine was determined by chemical analysis.

4. Paper electrophoresis: An apparatus designed in this laboratory was used.[8] It consisted of rectangular reservoirs of lucite which were enclosed on three sides by raised walls of lucite. When two reservoirs were apposed by approximating the slotted margins of their free side-walls and bases, a single enclosed rectangular chamber was formed which could be rendered air-tight by the addition of a peaked lucite cover. The "hanging strip" technique of Durrum (14) could then be employed by the insertion of a lucite rod through the overlapping side-walls above the two reservoirs. On the other hand, when the reservoirs were separated and two 33 by 38 by 2.5 cm. glass plates resting horizontally upon a wooden support were placed between them, paper electrophoresis by the technique of Kunkel and Tiselius (15) could be performed. Buffer capacity of each reservoir was two liters and the reservoirs were subdivided into communicating compartments in order to separate the carbon electrodes from the major portion of buffer.

Large sheets of Whatman No. 3 filter paper were employed for zone electrophoresis. The sheets were moistened with buffer and blotted prior to introduction into the electrophoresis apparatus. Two-hundredths milliliter aliquots of protein solutions were applied onto the sheets in straight 1.6 cm. lines separated from one another by marked intervals of 1.9 cm.

Since 33 by 50 cm. sheets were used in the Durrum system, nine specimens could be applied simultaneously. Applications were made directly upon the supporting rod or one inch beyond its anodal side. However, concurrent electrophoresis of multiple sheets was not feasible.

When the apparatus was adapted for electrophoresis by the method of Kunkel and Tiselius, 30 by 57 cm. sheets of filter paper were employed, thus limiting the capacity to eight specimens per sheet. Mobilities in all eight specimens were identical (Figure 1). Therefore, the "plate glass" system could be employed for simultaneous electrophoresis of multiple sheets. This modification offered certain advantages. For example, quadruplicate analysis could be readily secured by successively applying 0.02 ml. aliquots from eight specimens to four sheets which were directly superimposed upon one another. Furthermore, by interposing sheets of "Parafilm"[9] between successive sheets of filter paper, four sets of eight different solutions, or a total of thirty-two separate specimens could be electrophoresed at one time. The use of plate-glass 2.5 cm. in thickness obviated the need for equalizing pressure upon the filter paper by the use of clamps or other devices. Plates were covered with a thin coat of silicone[10] and cellophane tape was used to seal the sides of the plates.

Test solutions were delivered onto the filter paper with calibrated hemoglobin pipettes. Adsorption to glass was standardized by the use of a fresh pipette for each application. A small amount of powdered bromphenol blue was added to the site of delivery for visualization of albumin during electrophoretic migration.

Barbital buffer at pH 8.6, ionic strength 0.05 or acetate buffer at pH 4.5, ionic strength 0.03 to 0.10 was employed. To minimize changes in buffer pH, no buffer was used for more than a single experiment. In most studies, a potential of 117 volts was placed across the electrodes for twenty hours at room temperature. At pH 8.6, migrations averaged 12 to 15 cm. during this interval. Occasionally, voltages of 100 to 400 were employed for varying durations and maintained constant with a power supply and voltage regulator. Following electrophoresis, sheets were suspended in a drying rack in an oven at 130° C. for ten minutes.

5. Protein localization and radioactive assay: Staining prior to counting resulted in sufficient smearing and elution of counts to diminish the subsequent accuracy of radioactive assay. On the other hand, satisfactory staining could not be secured by reapposing the segments of strip which had been partitioned for counting. Therefore, in every experiment, electrophoresis was performed on duplicate sheets.

One sheet was directly radioautographed in contact with No-Screen X-ray film and subsequently stained according to the method of Durrum (14). Where indicated, protein distribution was quantitated with a densitometer.[11]

The second sheet was divided into component strips. These strips were further cut into 1 cm. segments beginning 1.5 cm. cathodal to the point of application and extending 1.5 cm. beyond the advancing albumin margin. Segments were counted in well-type scintillation counters containing crystal detector heads of thallium-activated sodium iodide.[12] A minimum of 3000 counts, with a

[8] Constructed by Transplastics Fabricating Co., 295 Huntington Ave., Boston, Mass.

[9] "Parafilm": Marathon Corporation, Menasha, Wisc.

[10] "Dri Film": General Electric, Silicone Products Department, Waterford, N. Y.

[11] Model 501 A, Photovolt Corporation, 95 Madison Ave., New York, 16, N. Y.

[12] Well-counters constructed by Macdonald Instrument Co., Cambridge, Mass. and N. Wood Counter Laboratory, Chicago, Ill.; strip scanner by Nuclear Instrument and Chemical Corp., Chicago, Ill.

probable error of 2 to 3 per cent, were secured in segments which approximated background; 10,000 counts above background were secured with segments containing higher radioactivity. In later experiments, intact strips were counted with an automatic strip scanner.[12]

Radioactivity in each segment (expressed as a percentage of the total radioactivity present on the strip) was plotted upon arithmetic graph paper. Areas under the radioactive peaks were delineated by techniques similar to those of conventional schlieren analysis. The percentile distribution of radioactivity was determined by weight. For final orientation, reference was made to the radioautographic and staining patterns of the specimens on the undivided duplicate sheet.

RESULTS

1. Methods

Potential errors resulting from methodological artifacts were evaluated.

a) Adsorption to glassware: In protein-free, saline solutions, there was considerable adsorption of thyroxine (both labelled and unlabelled) to glassware. Adsorption was maximal with carrier-poor solutions of radiothyroxine and varied inversely with the surface area of the beakers and pipettes which were employed during preparative dilutions. From saline solutions calculated to contain 5 to 200 μg. per cent of added thyroxine, 60 to 40 per cent of the radiothyroxine was adsorbed to glassware. In the presence of proteins capable of binding thyroxine, adsorption was negligible.

b) Recovery of radiothyroxine: Loss of radiothyroxine during electrophoretic migration was assessed by comparing the total counts on electrophoresed strips with similar aliquots which had been applied onto filter paper but not electrophoresed. After electrophoresis of protein solutions for six to thirty hours at pH 8.6 and 117 to 400 volts, the recovery of protein-bound radioactivity averaged 88.1 ± 13.4 [13] per cent in 152 observations with the "plate-glass system"; in 13 experiments with the Durrum method, recovery averaged 91.6 ± 10.9 [13] per cent. In 18 studies at pH 4.5, 84.5 ± 12.6 [13] per cent of the radioactivity could be accounted for in the area occupied by the proteins. Similar recovery experiments were performed with non-protein mixtures of thyroxine and saline. At pH 8.6, in 5 studies, 67.4 ± 2.6 [13] per cent of the radioactivity remained on the filter paper slightly anodal to the point of application; following electrophoresis of unbound thyroxine at pH 4.5, radioactivity remained principally at the origin and recovery averaged 65.5 per cent.

c) Recovery of inorganic I^{131}: Electrophoresis (pH 8.6, 117 volts, 18 to 20 hours) was performed following the addition of NaI^{131} to protein solutions. With serum or with saline solutions of albumin or Cohn Fraction IV-6, recovery of carrier-free inorganic I^{131} averaged 3.1 per cent; recoveries averaged 5.2 per cent when the protein solutions were supplemented with carrier to a final concentration of 200 mgm. per cent of inorganic iodide. In serum, the recovered radioactivity was principally confined to the albumin area. The data suggest that the presence of inorganic iodide (as a contaminant in the radiothyroxine or as a degradation product arising during electrophoresis) would not materially alter the electrophoretic partition of radioactivity.

d) Radiothyroxine: The electrophoretic distribution of other possible radioactive contaminants was not evaluated. For the following reasons, it seems unlikely that such impurities were present to any significant extent in fresh preparations of commercial radiothyroxine.

First, fresh radiothyroxine satisfied chromatographic criteria of homogeneity. Secondly, an identical partition of radioactivity between albumin and TBP was obtained when the specific activity of thyroxine-serum mixtures was varied five-fold and the total concentration of added thyroxine (*i.e.*, the sum of both labelled and unlabelled material) was maintained constant at 25 μg. per cent.

However, during storage *in vitro*, significant degradation of radiothyroxine occurs. To minimize the effects of ageing, only fresh preparations were employed. Moreover, potential errors from degraded moieties were kept constant by adding equal amounts of radiothyroxine to both the control and experimental specimens in all subsequent studies.

e) Reproducibility of radioactive assay: Serum was labelled with radiothyroxine and varyingly supplemented with stable thyroxine. Multiple aliquots of each specimen were electrophoresed simultaneously and analyzed separately for the partition of radioactivity. At PBI concentrations of 8 to 122 μg. per cent, the variability in the distribution of albumin and TBP-bound radioactivity

[13] Mean ± Standard Deviation.

in quintuplicate analyses was not greater than ± 5 per cent of the respective means for each category.

2. Validation of experimental design

Attempts to localize TBP in Cohn Fractions were based on the characteristics of the interaction between thyroxine and plasma proteins. Therefore, preliminary to an examination of Cohn Fractions, binding phenomena were studied in mixtures of serum and thyroxine.

a) Distribution of radiothyroxine: Sera from hyper-, hypo-, and euthyroid subjects were enriched with radiothyroxine and electrophoresed at pH 8.6. As others have shown (1–7), most of the labelled thyroxine localized with a moiety intermediate in mobility between the α_1 and α_2 globulins (Figure 1). A small but definite percentage of the radioactivity migrated with albumin even at the lowest concentrations of thyroxine. Eighty to 95 per cent of the radioactivity could be accounted for under the TBP and albumin peaks. Traces of radioactivity usually migrated ahead of the albumin area. The remaining radioactivity was varyingly distributed with the other anodal proteins and was concentrated principally in a narrow band, immediately ahead of the β globulins. Cathodal migration with γ globulin was never observed. In all sera, albumin-bound thyroxine progressively increased as the concentration of added thyroxine was augmented.

For the present, the significance of the apparent "spill-over" of thyroxine onto anodal components other than TBP or albumin cannot be assessed. Both in magnitude and pattern, it varied enormously. It could not be consistently augmented by increasing the concentration of thyroxine, although occasionally, it rose in parallel with the progressive elevation of albumin-bound radioac-

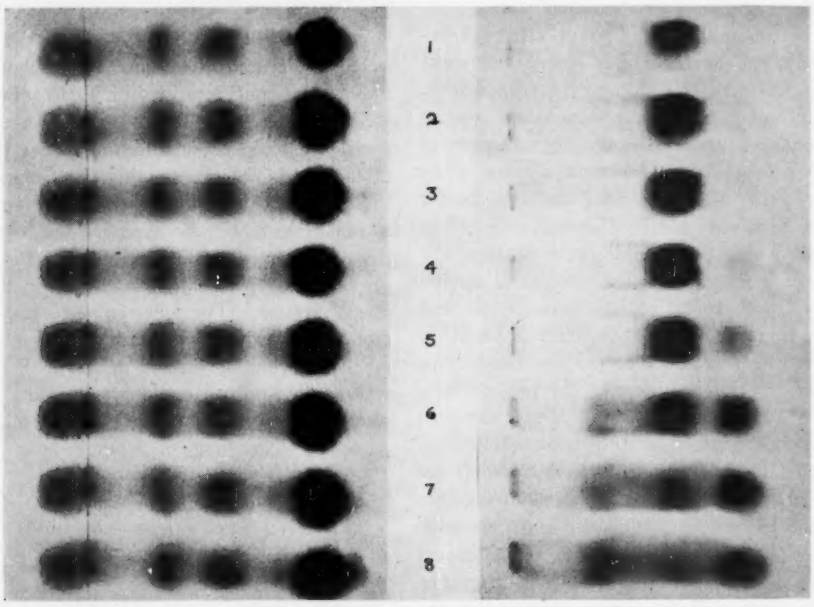

FIG. 1. PROTEIN BOUND RADIOACTIVITY AT VARIOUS CONCENTRATIONS OF THYROXINE

Serum from a myxedematous subject (PBI 1.4 μg. per cent) was labelled with radiothyroxine, and PBI concentrations of 3.2, 3.6, 6.7, 10.4, 19.3, 62, 112, and 194 μg. per cent were obtained in specimens 1 to 8, respectively, by the addition of unlabelled L-thyroxine. Following electrophoresis at pH 8.6, proteins were localized by staining with bromphenol blue. These are depicted on the left. The distribution of radioactivity is presented in the radioautographs on the right. The photograph does not clearly reproduce the definite amounts of albumin-bound radioactivity which could be discerned in specimens 1, 2, and 3 in the original radioautograph. As the PBI increases, progressive displacement of radioactivity from TBP to albumin occurs.

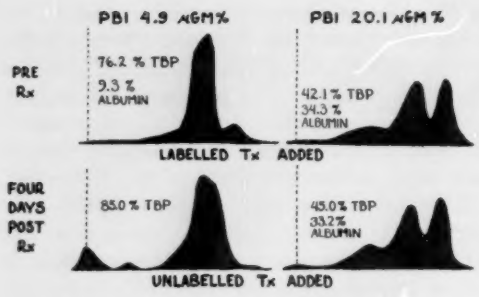

FIG. 2. EXCHANGE OF PROTEIN-BOUND THYROXINE

The upper two illustrations depict the distribution of radioactivity in serum to which radiothyroxine had been added *in vitro;* the serum shown in the lower illustrations was labelled endogenously with radiothyroxine which had been synthesized *in vivo.* In both sera, the concentrations or protein-bound iodine were adjusted by supplementation with stable L-thyroxine.

tivity (Figure 1). In some respects the phenomenon simulates the reported "trailing" due to adsorption of albumin during paper electrophoresis (16–18), and hence, it may merely represent methodological artifact. Despite these variations, the data indicate that albumin serves as the principal secondary carrier during the transport of thyroxine in serum.

b) Exchange of endogenous and exogenous thyroxine: Sera were obtained immediately before and four days after the administration of therapeutic doses of inorganic I^{131} for intractable angina pectoris. The thyroidal radiation was insufficient to liberate thyroglobulin into the circulation (19). Radiothyroxine was added to the non-radioactive sera obtained prior to therapy; the post-therapy sera were adjusted with stable L-thyroxine to identical concentrations of protein-bound iodine. Higher PBI levels were achieved by adding increasing amounts of stable thyroxine to aliquots of both the exogenously- and the endogenously-labelled sera. A typical experiment can be seen in Figure 2. At a PBI concentration of 4.9 μg. per cent, an apparent difference may be noted in the partition of radioactivity following exogenous and endogenous labelling of sera. This difference is probably not significant. Concentrations of radioactivity in endogenously labelled sera were considerably less than in sera labelled exogenously. In the former group, albumin-bound radioactivity was not detected by direct counting, but could be clearly discerned in radioautographs. Thus, the percentage of TBP-bound radioactivity in the endogenously labelled serum at a PBI 4.9 μg. per cent is probably artifactually increased.

c) Reversibility of albumin-binding: Various techniques were employed to demonstrate that the distribution of thyroxine between TBP and albumin conforms to a reversible binding equilibrium.

Serum was labelled with radiothyroxine; an aliquot of this serum was supplemented with stable L-thyroxine to yield a PBI level of 201.5 μg. per cent. Intermediate concentrations of protein-bound iodine were obtained by combining the enriched and the unenriched specimens in various proportions. The mixtures were electrophoresed and the distributions of radioactivity were quantitated. The results indicated that thyroxine which is bound to albumin can be recovered onto TBP by simply decreasing the PBI without changing the absolute concentration of either TBP or albumin (Table II).

By modifying conventional electrophoretic techniques, the concentrations of TBP, albumin, and PBI within a single serum could be altered independently or in combination to assess the effects of each variable upon binding phenomena.

Sufficient amounts of L-thyroxine were added to serum so that radiothyroxine was equally partitioned between albumin and TBP. Electrophoresis was performed at pH 8.6 in the "plate-glass" system. The albumin zone was detached from the less rapidly migrating components. Unlabelled serum of similar or lower PBI content was applied onto the albumin spot and a second electrophoresis was performed. In each instance, partial displacement of albumin-bound radioac-

TABLE II

The effect of PBI dilution on the distribution of radiothyroxine in plasma *

Specimen	PBI (μg. %)	% with TBP	% with albumin
1	6.5	87.2	7.7
2	12.6	80.6	11.5
3	18.4	74.8	16.8
4	30.9	69.9	25.3
5	55.2	46.9	41.9
6	104.0	28.5	50.3
7	201.5	24.5	49.6

* Specimens 1 and 7 mixed in varying proportions to obtain specimens 2–6.

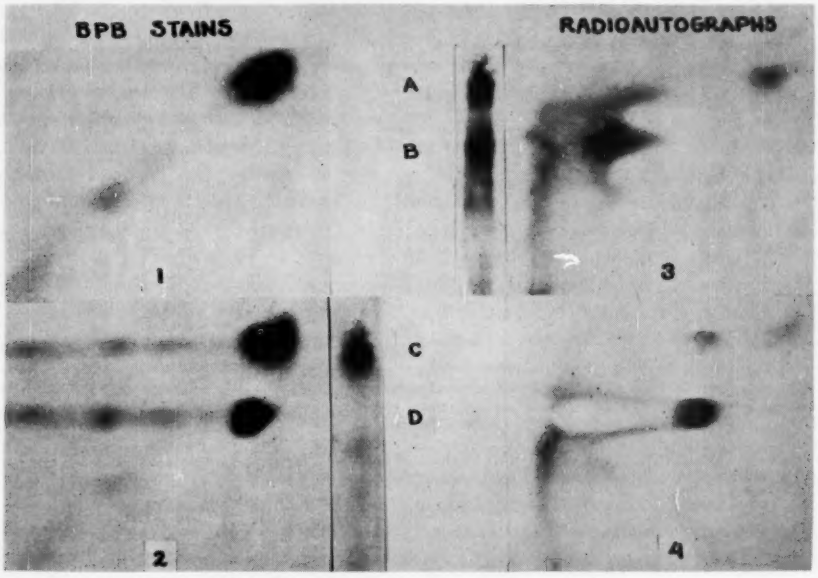

FIG. 3. TWO-DIMENSIONAL ELECTROPHORESIS

Illustrations 1 and 3 represent the respective distributions of proteins and radioactivity in a labelled specimen of serum which was electrophoresed in two dimensions. Illustrations 2 and 4 represent a similar two-dimensional electrophoresis in which additional unlabelled serum was applied at sites corresponding to albumin and TBP prior to electrophoresis in the second dimension. The latter manipulation was performed by localizing albumin (A and C) and TBP (B and D) with the aid of the two narrow strips of filter paper which are depicted in the center.

tivity onto the TBP of unlabelled serum could be demonstrated. No such partition occurred when the albumin was re-electrophoresed without the addition of unlabelled serum.

Similar results were obtained with "two-dimensional electrophoresis" (Figure 3). Herein, serum labelled with radiothyroxine was applied onto two sites spaced 13 cm. apart on a 26 by 57 cm. sheet of filter paper. For purposes of control, similar aliquots were applied onto two appended 2.5 by 57 cm. strips of filter paper. One dimensional electrophoresis was performed by the Kunkel-Tiselius method following which the appended strips were removed. One of them was radio-autographed. The second strip was rapidly dried and stained. With the stained strip for reference, protein moieties in the two bands on the large sheet of filter paper could be localized. Accordingly, the areas corresponding to TBP and albumin in one of these bands were supplemented with 0.020 ml. specimens of unlabelled serum. Electrophoresis in the second dimension was then performed by turning the entire sheet 90 degrees and by bridging it to the buffer reservoirs with fresh filter paper. It was found that TBP of the added unlabelled serum could abstract most of the radioactivity which, initially, had migrated with albumin. On the other hand, a shift in the distribution of radioactivity was not observed in the control band which had not been supplemented with serum prior to the second electrophoresis.

d) Conclusion: In serum, endogenous and exogenous thyroxine are in ready exchange; thyroxine is distributed among plasma proteins according to a reversible binding equilibrium between TBP and secondary carriers of lesser affinity; displacement onto secondary carriers (principally albumin) occurs with increasing thyroxine concentrations; and displaced thyroxine can be recovered onto TBP by decreasing the concentration of thyroxine or by increasing the availability of TBP.

TABLE III

The effect of added Cohn Fractions on the distribution of radiothyroxine in serum

Fraction	Fractions from fresh plasma		Fractions from old plasma	
	% with TBP	% with alb.	% with TBP	% with alb.
Serum control	42.6 ± 3.0 (7)*	44.4 ± 2.1 (7)*	34.2 ± 0.9 (5)†	49.3 ± 2.9 (5)†
Serum + I	40.9	44.9	32.4	54.1
Serum + II–III	42.1	43.9	34.3	51.7
Serum + IV-1	45.6	44.7	34.5	55.6
Serum + IV-5	57.9	31.5	58.9	30.3
Serum + IV-6	78.8	15.0	74.4	18.0
Serum + IV-7	54.3	37.0	47.2	41.4
Serum + IV-8	55.7	34.3	51.8	39.0
Serum + IV-9	65.6	26.6	61.4	30.4
Serum + V	43.8	47.2	33.5	54.6
Serum + Super. V	39.8	46.7	32.9	53.6

* The concentration of PBI in the serum of an euthyroid subject (J. P.) was adjusted to 50.0 μg. per cent. The figures in parentheses denote the number of simultaneous electrophoreses which were performed to derive standard deviations for the distribution of radioactivity prior to the addition of Cohn Fractions which had been obtained from fresh plasma.

† The concentration of PBI in the serum of an euthyroid subject (N. F.) was adjusted to 46.6 μg. per cent. Added Cohn Fractions were prepared from outdated blood-bank blood.

3. Cohn Fractions

The foregoing observations formed the basis for testing Cohn Fractions.

a) Mixtures of Cohn Fractions and serum: Labelled and unlabelled thyroxine were added to sera to yield concentrations of protein bound iodine ranging from 26 to 90 μg. per cent. At these PBI levels, radiothyroxine was varyingly partitioned between TBP and albumin. Twenty-seven mgm. amounts of lyophilized Cohn Fractions were dissolved in 1 ml. aliquots of the sera;[14] no attempt was made to correct for insoluble materials. Concurrent paper electrophoreses were performed with sera containing added Cohn Fractions (*circa* 2.7 Gm. per cent) and with at least four control specimens to which extra protein had not been added. It was anticipated that albumin-bound thyroxine might be recovered from albumin onto TBP by Cohn Fractions containing ratios of TBP/albumin greater than the normal serum proportions.

The partition of radioactivity was not altered by Cohn Fractions I, II–III, IV–I, V, and Supernate V[15] (Table III). However, all sub-groups of Fraction IV-4 (*i.e.*, IV-5, IV-6, IV-9; and IV-7, IV-8) significantly diminished the radioactivity in the albumin area and enhanced the apparent binding of thyroxine by TBP. Fractions IV-6 and IV-9 produced the most pronounced augmentation of TBP activity. Age of the parent plasma did not affect results. Findings were similar with Cohn Fractions derived from outdated blood bank plasma as well as from fresh plasma (Table III).

b) Mixtures of Cohn Fractions and saline: Lyophilized Cohn Fractions were dissolved in isotonic saline[14] and repeatedly centrifuged to remove all insoluble denatured material. Biuret analyses were performed to adjust the protein concentration in all solutions to 2.0 gram per cent. Control specimens of serum were diluted to a comparable extent. At pH 8.6, the components of the various Cohn Fractions retained their characteristic electrophoretic mobilities. Thus, for purposes of reference, the migration of radioactivity could be related to simultaneously electrophoresed specimens of serum.

[14] Heparin was added to prevent clotting in all experiments involving Cohn Fraction I.

[15] Currently, Fraction VI is concentrated with zinc hydroxide (28) from the supernatant solution which remains after the precipitation of Fraction V according to the low temperature-low salt-ethanol fraction method (8). Preliminary evidence would indicate that zinc reduces the quantity of thyroxine bound by TBP (20). Therefore, attempts were made to obtain the proteins of Fraction VI without zinc precipitation.

Excess salts and ethanol in Supernate V were removed by dialysis against repeated changes of distilled water at 4° C. The dialyzed Supernate V was lyophilized and the recovered proteins were employed in subsequent experiments. Similar dialysis and lyophilization did not diminish the binding of thyroxine by Cohn Fraction IV-6.

Studies were designed to demonstrate trace amounts of thyroxine-binding proteins and to compare their relative abundances on the basis of saturation characteristics. Low concentrations of carrier thyroxine were employed to meet the first objective. The PBI concentration in all solutions was adjusted to 11.4 μg. per cent with stable and labelled L-thyroxine. Paper electrophoresis at pH 8.6 was performed and the distribution of radioactivity was quantitatively assessed (Figure 4, Column 1). Radioactivity in saline solutions of Cohn Fractions I and II-III extended from the origin to a zone immediately ahead of the β globulins. Contrariwise, some of the radioactivity in Fractions IV-1, IV-7, V and Supernate V, and all of the radioactivity in Fractions IV-5, IV-6, IV-8, and IV-9 migrated in parallel with the TBP of serum. Staining with bromphenol blue was not appreciable in any of these areas of concentrated radioactivity.

For comparison of relative binding capacities, the concentration of protein-bound iodine in all solutions was augmented to 71.4 μg. per cent with unlabelled thyroxine (Figure 4, Column 2). Such loading did not alter the diffuse distribution of radioactivity in solutions containing either Cohn Fractions I or II-III. On the other hand, in the control sample of serum, most of the radioactivity was displaced from the TBP onto albumin. Similarly, in all Cohn Fractions other than I and II-III, the addition of large amounts of thyroxine caused partial or complete "spill-over" of radioactivity onto secondary carriers. Proteins other than albumin served as secondary carriers in fractions which were deficient in albumin. Displacement was least in solutions containing IV-6 or IV-9 and most of the radioactivity remained confined to a discrete zone corresponding in electrophoretic mobility at pH 8.6 to the TBP of serum.

The specificity of thyroxine-binding in IV-6 and IV-9 was assessed by electrophoresis in acetate buffer at pH 4.5 (Figure 5). At this pH, free radiothyroxine or radiothyroxine combined with Cohn Fraction II-III remained at the origin. Labelled TBP in thyroxine-serum mixtures migrated towards the anode in confirmation of the report of Robbins, Petermann, and Rall (21). The radioactivity in solutions containing Fractions IV-6 and IV-9 also migrated slowly towards the positive pole despite the predominant cathodal movement of most of the other proteins.

c) Mixtures of Cohn Fractions, saline and

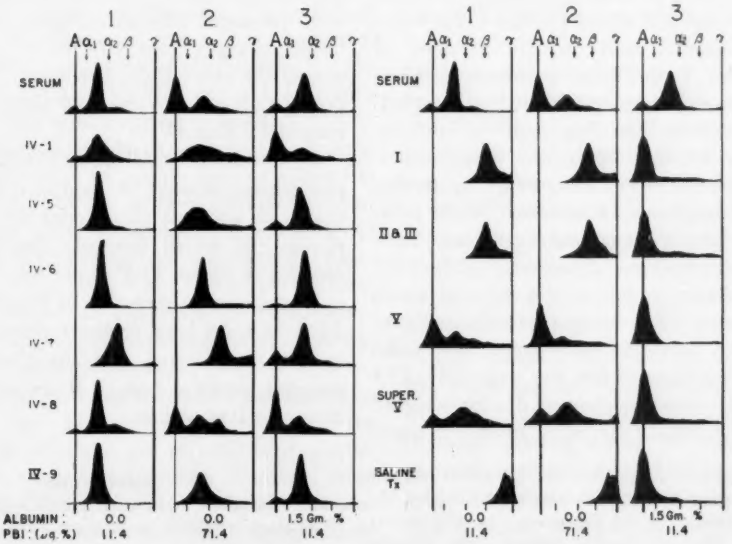

FIG. 4. THYROXINE BINDING BY COHN FRACTIONS

The darkened areas represent the distribution of radioactivity following paper electrophoresis.

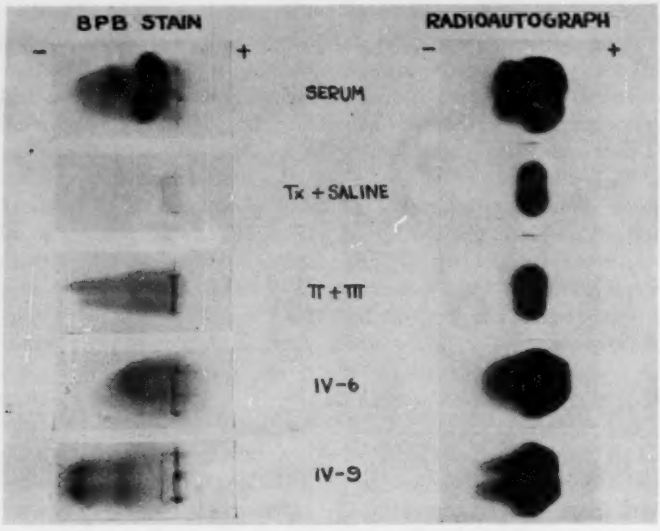

FIG. 5. ZONE ELECTROPHORESIS AT pH 4.5

The similarities in the anodal migrations of serum TBP and the thyroxine-binding moieties in Fractions IV-6 and IV-9 can be seen in the radioautographs on the right.

mercaptalbumin: To simulate the inter-relationships of proteins in serum and to compensate for the variable content of albumin in Cohn Fractions, attempts were made to electrophorese Cohn Fractions in association with constant amounts of added albumin. It was hoped that such combination might provide further quantitative comparison of the thyroxine-binding moieties in Cohn Fractions. Albumin preparations whose homogeneity ranged from 94 to 100 per cent by moving boundary electrophoresis were tested for the presence of non-albumin contaminants capable of binding thyroxine. Only mercaptalbumin (22) [16] did not contain detectable traces of a material which resembled serum TBP during paper electrophoresis at pH 8.6. Therefore, saline solutions of Cohn Fractions (Protein: 1.85 Gm. per cent; PBI: 11.4 μg. per cent) were supplemented with weighed amounts of lyophilized mercaptalbumin (15 mgm. per ml.) prior to paper electrophoresis at pH 8.6. All of the radioactivity in solutions containing Fractions I, II-III, V, and Supernate V migrated with the added albumin (Figure 4, Column 3). Lesser changes were effected in mixtures containing the other Cohn Fractions. The distribution of radioactivity was least altered in the presence of IV-6 or IV-9.

d) Conclusions: All Cohn Fractions which are precipitated subsequent to Fraction III contain detectable amounts of material which binds thyroxine and which simulates the electrophoretic mobility of serum TBP at pH 8.6. This material is most abundantly present in Fractions IV-6 and IV-9. On the basis of electrophoresis at pH 4.5, it may be inferred that the thyroxine-binding moieties in IV-6 and IV-9 are indeed the same as the TBP of serum.

DISCUSSION

Earlier attempts to localize TBP in Cohn Fractions of plasma have followed several lines. By dialysis, it has been shown that thyroxine-binding is minimal with gamma globulin (23). However,

[16] Mercaptalbumin which had been dialyzed against cysteine to remove mercury was kindly provided by Dr. Margaret J. Hunter of the Department of Biophysical Chemistry of Harvard Medical School. The albumin preparations which contained TBP-like impurities included crystalline bovine albumin and various reworked preparations of Fraction V.

since thyroxine is bound, at least partly, by all proteins, the dialysis technique affords limited specificity. Other investigators have relied upon measurements of iodine/nitrogen ratios (23, 24). With this approach, Salter meticulously localized the thyroxine-transport protein in Fractions IV and V, and even succeeded in specifically implicating the subfractions of IV which were rich in alpha globulins (*i.e.*, IV-5 and IV-6) (25). However, the differences among various fractions were small and inconsistent, and it is likely that some of the variability may have resulted from artifacts intrinsic to the fractionation technique. During fractionation by Method 6 of Cohn, disruption of thyroxine-protein bonds and reagent contamination may progressively increase the content of unbound inorganic and organic iodine in the alcohol-protein-electrolyte mixtures. That coprecipitation and occlusion of these iodine moieties does occur is suggested by the routine demonstration of significant amounts of protein-bound iodine in fractions which do not contain detectable amounts of TBP such as Fractions I or II-III (23–25). Errors thereby introduced would become especially manifest in fractions which are precipitated last, either in great bulk (*cf.* Fraction V) or in small amounts from large volumes of suspending solution (*cf.* Fraction VI). It is of interest in this connection that high iodine/nitrogen ratios have been reported for Fraction V (23–25) and for Fraction VI (25, 26) although their content of TBP would appear to be small.

Obviously, it would be most desirable to measure TBP directly. As yet, no such technique has been evolved. In the present experiments, attempts were made to minimize the difficulties which are inherent in indirect approaches. Fractions prepared by Methods 6 of Cohn were compared by a variety of saturation and competitive-binding techniques; zone electrophoresis was employed to provide specificity.

It may be argued that saturation characteristics cannot be used for intercomparisons since the fractionation technique, *per se*, may varyingly desaturate or remove endogenous thyroxine from the different Cohn Fractions. Although this possibility cannot be excluded, it would seem unlikely that variation in endogenous hormone (*e.g.*, iodine/protein ratios; Table I) could significantly affect the results obtained in systems wherein the concentration of added carrier thyroxine was greater than 100 μg. per cent (v.s.).

Certain properties of TBP were noted in the course of the present experiments. It may be inferred that the TBP of Fraction IV-6 is not a lipoprotein; multiple freezings and thawings did not alter its binding characteristics. TBP is highly soluble in water, and neither solubility nor thyroxine-binding were diminished by dialyzing IV-6 against repeated changes of distilled water to remove occluded inorganic moieties. The stability of TBP is evidenced by the observation that fractions derived from fresh and from outdated blood did not appreciably differ in activity.

Zinc interferes with the interaction of thyroxine and TBP in serum and in Fractions IV-6 and IV-9. Conceivably, therefore, the techniques described above may not be applicable to proteins which have been prepared by zinc precipitation. Among these proteins may be included the products of Method 10 of Cohn and his co-workers (*27*) and the pure α glycoproteins of Schmid (28, 29).

Throughout this report, the term TBP has been employed to designate the thyroxine-binding protein of plasma. It should be stressed that nomenclatural selection of the singular is arbitrary and intended solely for convenience. By labelling the TBP in serum with radiothyroxine, Petermann, Robbins, and Hamilton have derived an ultracentrifugal sedimentation constant for TBP (30), and Robbins, Petermann, and Rall (21) have shown that the electrophoretic mobility of TBP at pH 4.5 simulates that of the M-2 glycoproteins of Mehl, Golden, and Winzler (31). However, to date, TBP has not been isolated. Thus, despite all physicochemical measurements, it cannot be said whether TBP represents only one protein or several proteins which, in protein mixtures, exhibit similar electrophoretic and ultracentrifugal characteristics. Furthermore, if TBP is a single protein, the possibility of multiple sites for thyroxine-binding cannot be excluded. Because of these considerations, the present data have been subjected only to descriptive analysis. It was felt that available information did not justify more quantitative treatment, and that precise stoichiometrical formulations must await the availability of pure TBP.

SUMMARY

The interactions between thyroxine and plasma proteins have been investigated with paper electrophoresis. The observations furnished an experimental basis for multiple techniques designed to localize the thyroxine-binding protein (TBP) in fractions of plasma prepared by Method 6 of Cohn. It has been demonstrated that Cohn Fractions IV-6 and IV-9 constitute the most abundant sources of TBP.

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THE EFFECT OF GLYCOGEN DEPOSITION ON LIVER PHOSPHORUS [1, 2]

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It has been a common concept that the deposition of glycogen within liver cells is accompanied by an accretion of water, potassium, and phosphate from outside the liver. The purpose of this paper is to show that glycogen deposition is indeed accompanied by the accretion of water and potassium, as originally demonstrated by Fenn (1), but that no phosphate enters the liver during glycogen deposition. We have found that the cells of livers low in glycogen contain more phosphate than can be completely ionized in the cell water if electroneutrality and osmotic equilibrium are to be maintained. As glycogen levels rise in the cells, accompanied by the accretion of water and potassium the amount of this excess phosphate diminishes in a fashion mathematically consistent with dilution. We therefore postulate that the anion equivalency and osmotic force required to maintain electroneutrality and osmotic equilibrium in the intracellular water that is deposited with glycogen are supplied by the conversion of acid-insoluble phosphate already present in the liver, to acid-soluble phosphate esters and orthophosphate.

METHODS

The data presented were obtained from the livers of 15 rabbits. Four groups of rabbits were included: normal rabbits, alloxan-diabetic rabbits, normal rabbits which had received cortisone (7.5 mg. for 16 to 18 days) and diabetic rabbits which had received cortisone.

Following intravenous Nembutal® anesthesia, the animals were exsanguinated, and their livers removed *in toto*. The livers were weighed and one sample placed immediately in 30 per cent KOH for glycogen determination. A second sample was placed in a weighing bottle for the determination of fat, water, nitrogen, and electrolytes.

Serum water was determined using a micropyknometer. Serum sodium and potassium were measured on an internal standard flame photometer. Chloride was determined by the method of Wilson and Ball (2). The Somogyi-Nelson method (3) was used for the determination of blood sugars. Inorganic serum phosphate was measured by the molybdenum-blue reaction as described by Fiske and Subbarow (4). A modification of Folin's method (5), using Nessler's reagent, was used in the determination of non-protein nitrogen.

The livers were analyzed for water by drying to constant weight at 100 to 102° C. Neutral fat was determined by extracting the powdered dry livers three times with a mixture of equal parts of ethyl and petroleum ether (6). Sodium, potassium, and chloride analyses were carried out on aliquots of a 0.75 N redistilled nitric acid extract of dry fat-free tissue (7). Sodium and potassium were determined by internal standard flame photometry and chloride by a micro-modification of the method of Wilson and Ball (2). For the determination of total phosphate, samples of dry fat-free liver powder were predigested with concentrated nitric acid, followed by perchloric acid until a clear, colorless solution was obtained. This solution was transferred quantitatively to a volumetric flask and aliquots used for the determination of total phosphate by the molybdenum-blue reaction. Total nitrogen was measured on an aliquot of tissue which was predigested with sulfuric acid and superoxol. The resulting clear, colorless solution was cooled, transferred quantitatively to a 25 ml. volumetric flask, and made to volume. Aliquots of this solution were further digested in a sulfuric-phosphoric mixture and the nitrogen determined by Nesslerization.

Calculations

Determination of the intracellular concentrations of phosphate, potassium, and sodium was made using the chloride space as a measure of extracellular water, as described by Hastings and Eichelberger (6). The application of this calculation assumes that the intracellular phase of the tissue contains no chloride, an assumption that is probably correct under physiological conditions, but may be incorrect under conditions of abnormal metabolism. The following equations present the method of calculation.

All analyses are calculated on fat-free tissue values.

$(Cl)_s$ = mEq. Chloride per liter serum.

$[Cl]_w$ = mEq. Chloride per kilogram serum water.

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[2] Published in abstract form in the proceedings of the 46th Annual Meeting of the American Society for Clinical Investigation, J. Clin. Invest., 1954, 33, 956.

$[Cl]_e$ = mEq. Chloride per kilogram extracellular water.
$(Cl)_K$ = mEq. Chloride per kilogram liver.
$(H_2O)_s$ = Grams water per liter serum.
$(H_2O)_K$ = Grams water per kilogram liver.
$(H_2O)_e$ = Grams extracellular water per kilogram liver.
$(H_2O)_i$ = Grams intracellular water per kilogram liver.
$(I)_K$ = mEq. ion per kilogram liver.
$(I)_e$ = mEq. ion per extracellular phase of a kilogram of liver.
$(I)_i$ = mEq. ion per intracellular phase of a kilogram of liver.
$[I]_i$ = mEq. ion per kilogram of intracellular water.
0.96 = Donnan factor.

(1) $\frac{(Cl)_e}{(H_2O)_e} = [Cl]_e$ (4) $(H_2O)_K - (H_2O)_e = (H_2O)_i$

(2) $\frac{[Cl]_e}{0.96} = [Cl]_e$ (5) $(I)_K - (I)_e = (I)_i$

(3) $\frac{(Cl)_K}{[Cl]_e} = (H_2O)_e$ (6) $\frac{(I)_i \times 1000}{(H_2O)_i} = I_i$

Definitions

The following definitions are used throughout this paper in discussing phosphate. They are based on Umbreit, Burris, and Stauffer's classification (8).

Total phosphate: All of the phosphate present in the tissue (the sum of acid-soluble and acid-insoluble phosphate).

Inorganic (ortho) phosphate: Phosphate that is present as the dissociation products of phosphoric acid. This phosphate is not in combination or equilibrium with any organic compound.

The dissociation constant for the over-all equilibrium of phosphoric acid at pH 6.9 is taken to be 1.3 (9).

Acid-soluble phosphate: Phosphate that is soluble in 10 per cent trichloracetic acid. This includes inorganic phosphate, phosphate esters such as the glucose phosphates which, in solution yield orthophosphate, and phosphate esters such as ATP and ADP, which are pyrophosphates and, on dissociation may not yield orthophosphate. Since the pKs of all of the orthophosphate esters are lower than that of phosphoric acid, and the pKs of ATP and ADP are similar (10), it follows that the dissociation constant of acid-soluble phosphate as a whole must be at least 1.3, and may be greater.

Acid-insoluble phosphate: Phosphate that is insoluble in 10 per cent trichloracetic acid, is bound to protein, and is presumably not ionized in cell water. This phosphate includes phospholipids and nucleoproteins.

RESULTS

Table I presents the basic data obtained. The livers of the animals receiving cortisone were markedly increased in size, both in normals and diabetics, and contained high concentrations of glycogen. As the glycogen concentrations in these livers increased, the concentration of total phosphate fell, while the concentrations of water and potassium remained relatively constant. Nitrogen concentrations fell as glycogen increased. Sodium and chloride showed no changes that paralleled the increase in glycogen content. Serum data showed normal values in all animals, with the exception of elevated blood sugars in diabetic animals.

Figure 1 illustrates the inverse relationship between total phosphate (as mM per 100 Gm. fat-free dry solids) and glycogen (Gm. dry glucose per 100 Gm. fat-free dry solids) found in these livers. The line was plotted from the formula of least squares and indicates that as the concentration of glycogen increases, the concentration of total phosphate decreases. In a liver with a concentration of 10 Gm. of glycogen per 100 Gm. FF dry solids there is a concentration of 45 mM of phosphate. As the concentration of glycogen increases, total phosphate falls, reaching at a level of 50 Gm. of glycogen, a value of 26.4 mM. The correlation coefficient for this line is 0.9564, with a z value of 1.90 ± 0.29, showing it to be highly significant.

DISCUSSION

From the data presented in Table I and Figure 1, it can be seen that, as glycogen is deposited in the liver, the concentration of total phosphate falls in a straight line, consistent with dilution.[3] Calculation of the intracellular concentration of phosphate in the liver with very low glycogen or high glycogen content, reveals that were all of the phosphate that is present in the tissue electrically and osmotically active in the intracellular water, neither electroneutrality within the cell or osmotic equality between intracellular and extracellular water would exist. Table II illustrates this.

This table presents the values for the calculated intracellular concentrations of potassium, sodium, and phosphate in a rabbit with a very low concentration of liver glycogen (No. 12), and a rabbit

[3] If a liver with 10 Gm. glycogen per 100 Gm. fat-free dry solids contains 45 mM of phosphate per 100 Gm. fat-free dry solids, then, if no phosphate is added to the liver as glycogen is deposited, the predicted phosphate concentration for a liver with 40 Gm. glycogen per 160 Gm. fat-free dry solids would be $49 \div 1.3$ or 34.6 mM of phosphate per 100 Gm. fat-free dry solids. The actual value found, taken from Figure 1 is 30.6 mM per 100 Gm. fat-free dry solids.

TABLE I

LIVER

Basic Data

Rabbit No.	Group	Liver Weight Gms.	% Body Weight	Neutral Fat Gms.	H_2O Gms./100 Gms. FF	Protein Dry Solids	Glycogen Dry Solids	Total Phosphate mM/100 Gms. F F Dry Solids	Potassium mEq/100 Gms. FF	Chloride Dry Solids	Sodium Dry Solids
12	D	193	3.0	22	296	88.8	6.9	44.7	34.1	9.1	18.0
48	N	152	3.7	14	270	72.8	19.9	39.5	32.3	8.6	15.0
31	N	142	3.6	16	274	74.8	21.8	41.2	33.6	8.1	13.0
32	N	175	3.4	15	262	76.8	29.6	38.3	33.4	7.8	13.7
33	D	117	3.5	13	260	63.3	33.8	32.0	34.5	10.9	17.9
3	D	135	3.6	12	265	57.8	34.2	32.6	31.3	7.6	17.3
37	N	156	4.2	18	261	65.2	36.1	32.0	31.3	10.5	14.3
34	D + C	205	6.6	20	260	51.2	36.8	32.7	30.4	7.6	12.6
41	N + C	244	7.6	51	262	53.8	42.2	29.7	33.3	8.2	14.8
27	D + C	415	11.5	17	262	45.8	42.6	25.2	29.9	7.2	10.7
2	D + C	300	9.5	28	252	53.2	43.9	27.1	29.8	6.5	11.5
1	N + C	224	7.3	18	258	48.5	48.7	27.6	33.8	7.5	14.5
17	D + C	242	7.2	17	268	48.5	49.8	28.3	27.9	6.2	11.7
40	N + C	273	8.4	21	254	45.8	51.2	26.4	29.0	7.1	14.3
100	D + C	350	9.8	47	253	47.2	51.5	25.3	28.7	5.3	10.6

SERUM

Average of 15 Rabbits

	H_2O Gms./L	Na	K	Cl	PO_4
		mEq/Liter			mgm/%
	942	147	5.5	101	6.7
S.D.	5.77	2.89	0.16	5.4	0.89

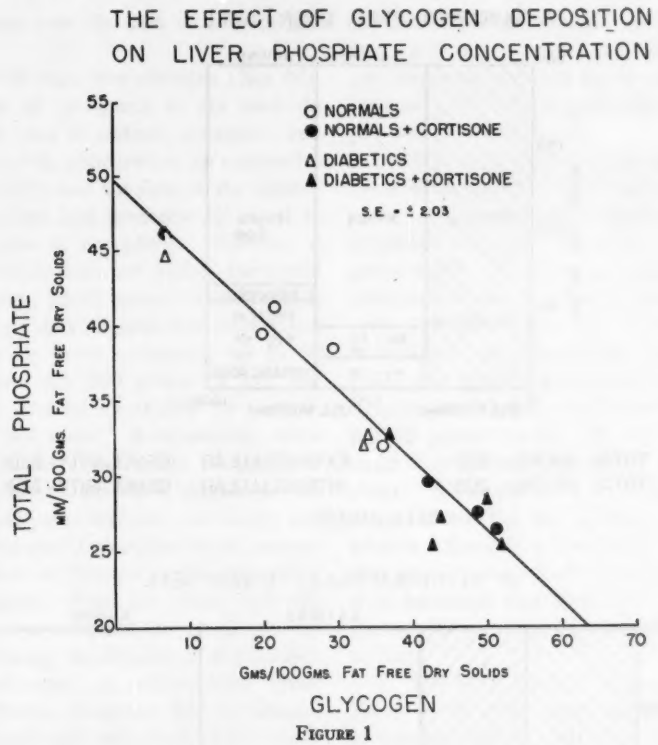


FIGURE 1

with a very high concentration of liver glycogen (No. 40), making the assumption that all liver phosphate is osmotically and electrically active. In the case of the rabbit with very low liver glycogen, there is a total of 397 milliosmols present in the intracellular fluid, as opposed to 320 in the extracellular fluid. (This does not take into account the additional milliosmols that must be present as magnesium, bicarbonate, sulfate, protein and organic acids.) Furthermore, the sum of the anions exceeds the sum of the cations. Assuming a dissociation constant of 1.3 for phosphate, there are 276 milliequivalents of phosphate present in association with 185 milliequivalents of sodium and potassium. Probably phosphate has a higher base-binding capacity than is indicated, because of the lower pK of carbohydrate esters. However, even if we assume a valence of one for phosphate, and assume that bicarbonate, protein sulfate and organic acids contribute no negative charges to the cell water, we still have 212 milliequivalents of phosphate present in the cell water—an excess of

TABLE II

Rabbit No. 12

Liver Glycogen = 6.9 Gm./100 Gm. FF Dry Solids

	mOsm.	*mEq.*
Potassium	161	161
Sodium	24	24
Phosphate	212	276 (f = 1.3)

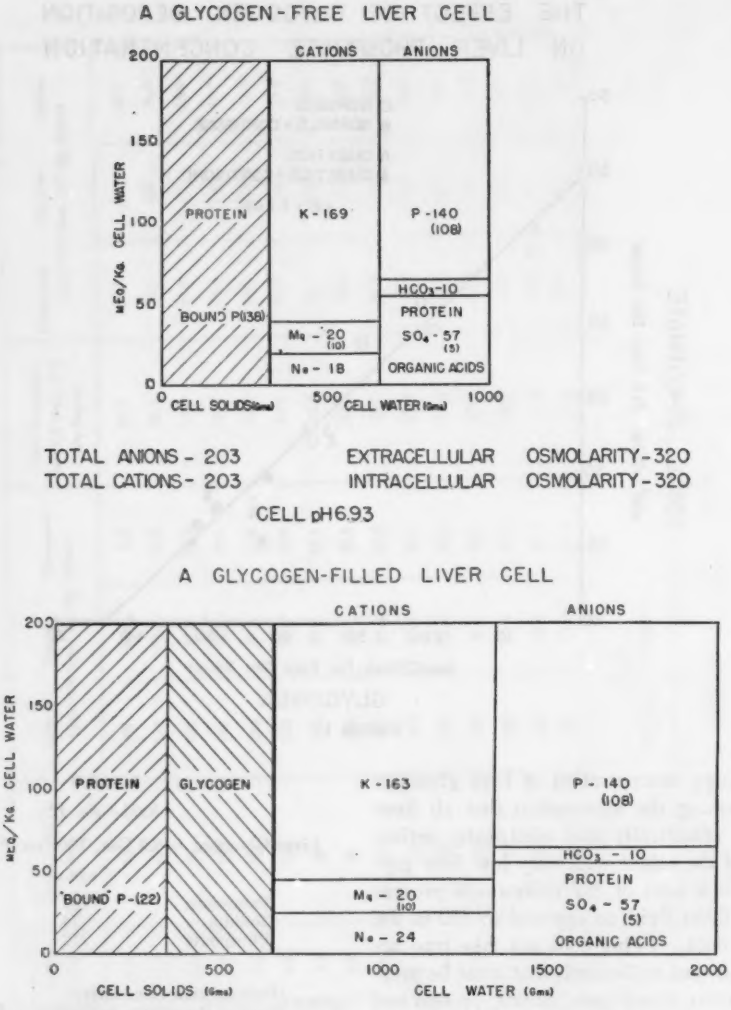
mOsm.	Extracellular Osmolarity	320
	Sum of Potassium + Sodium + Phosphate	397
mEq.	Sum of Sodium and Potassium	185
	Phosphate	276

Rabbit No. 40

Liver Glycogen = 51.2 Gm./100 Gm. FF Dry Solids

	mOsm.	*mEq.*
Potassium	146	146
Sodium	28	28
Phosphate	132	172 (f = 1.3)

mOsm.	Extracellular Osmolarity	320
	Sum of Potassium + Sodium + Phosphate	306
mEq.	Sum of Sodium and Potassium	174
	Phosphate	172



FIGURES 2 AND 3

1. The amount of *intracellular water* in a kilogram of cells is calculated as shown in the definitions (see Equation 5). The data used in Figures 2 and 3 is the average of the values for the 15 rabbits.

2. All of the *solids* (as calculated from wet weight − dry weight, corrected for fat content) are assumed to be intracellular. The data used in Figures 2 and 3 are the average value for the grams of solids associated with one kilogram of cells in the 15 rabbits.

3. Values for the concentration of *intracellular ions* were calculated from the equations presented.

4. *Potassium and sodium.* Figure 2: The average of the values for the two normal rabbits having the lowest liver glycogens (48 and 31). Figure 3: The average of the values for the two normal rabbits receiving cortisone having the highest liver glycogen (I and 40).

27 milliequivalents over the sum of sodium and potassium.

In the rabbit with high liver glycogen (No. 40), this large excess of phosphate is not seen—in fact, the osmolar sum of sodium, potassium and phosphate is only 306, compared to an extracellular osmolarity of 320, and the sum of the equivalent value of sodium and potassium is equal to the equivalent value of phosphate. However, if the other intracellular ions are added, there will again be a definite, small excess of milliosmols and anions. These data suggest that more phosphate is present in livers containing up to 50 grams of glycogen per 100 grams of dry fat-free solids than can be electrically or osmotically active in cell water. Furthermore, since there is no addition of phosphate to the liver during the deposition of glycogen, despite the addition of water and potassium, the osmotically and ionically active fraction of phosphate must increase with the deposition of glycogen at the expense of the bound phosphate. Fenn has shown (1) that the concentration of acid-soluble phosphate remains constant during the deposition of glycogen, while the concentration of acid-insoluble phosphate declines. Hence, it appears that the demand for more osmotically and electrically active phosphate ions during glycogen deposition is met by the conversion of acid-insoluble, or bound, phosphate, to acid-soluble phosphate esters and inorganic orthophosphates.

In contrast to the qualitative data presented in Table II, Figures 2 and 3 have been constructed to present a quantitative picture of the effect of glycogen deposition on the intracellular composition of liver. Certain assumptions necessary to the quantitative construction of these diagrams are presented below. The source of the data is indicated in the legend.

Assumptions

1) An intracellular pH of 6.9 was assumed throughout the calculations. This figure is based on data for muscle and may be inaccurate for liver, although the data of Cori and Cori on the pH limits for the activity of specific liver phosphatase (pH 6-7) suggest that the intracellular pH lies close to 7.0 (12).

2) Acid-soluble intracellular phosphate has been assumed to have an anion equivalence of 1.3 per mole. This is calculated from the dissociation curve of phosphoric acid (9) and is, at best, a rough approximation. We do know that most acid-soluble phosphate esters have pK's which are lower than orthophosphate (10) and, therefore, the anion equivalency of intracellular acid-soluble phosphate may actually be greater than 1.3. Without quantitating all of the acid-soluble phosphate compounds present in the cell, one cannot derive the exact base-binding capacity of intracellular phosphate. Furthermore, it is at present impossible to quantitate the base-binding capacities of cell protein. Since it is the sum of these two anions that provide almost all of the anions in the cell, it is inevitable that there will be some error in the specific number of acid equivalents assigned to each.

3) The total amount of magnesium found in livers is in close agreement between different groups of workers (13, 14). However, there is little evidence concerning the ionization of liver magnesium. What few data there are favor its being bound in part (15, 16). For this reason, we have assumed that only two-thirds of the total magnesium in liver (10 of 15 milliosmols per Kg. intracellular water) exists in a free form. We have also assumed that magnesium is added to liver cells during the deposition of glycogen. There is no experimental evidence yet on this subject.

4) Very little is known about the intracellular concentrations of protein, sulfate, and organic acids. However, it is generally conceded that minor amounts of these substances do occur within cells (17, 18), contributing a small number of milliosmols (about 5) and a greater number of

5. *Magnesium.* The average value found by a number of workers is 15 millimols per kilogram of intracellular water. A figure of 10 millimols, with an anion equivalency of 20, was used in the construction of these diagrams, with the assumption that some of the magnesium is bound to cell protein, and is not in solution in the intracellular water.

6. *Phosphate.* The values for phosphate were taken from Figure 1. The figure 49.8 mM of phosphate per 100 Gm. of fat-free dry solids, used in the construction of Figure 2, was taken from extrapolation of the line to zero. Twenty-four mM per 100 Gm. of fat-free dry solids was the figure used to calculate the intracellular concentration of phosphate in the liver where glycogen constitutes 50 per cent of the solids.

7. *Bicarbonate.* From the data of Wallace and Hastings (11).

8. *Protein, sulfate, and organic acids.* From the data of Hastings (17) and Gamble (18).

milliequivalents (about 50) to intracellular fluid. (The large discrepancy between the osmolar and anionic value is due to the polyvalency of the protein molecules.) Their molecular concentration in intracellular fluid is so low that large errors in estimation are of little importance in the total osmotic sum. Errors in the estimation of the anionic equivalence of protein may be present, as phosphate may have a greater anionic equivalence than 1.3, as has been discussed.

Figures 2 and 3 each represent a liver cell. For the sake of simplicity, the cell in Figure 2 has been assumed to weigh 1000 Gm. This is indicated on the abscissa. The concentrations of electrolytes present (per Kg. of intracellular water) are plotted along the ordinate and are also marked in the cells. Where the osmolar value of an electrolyte differs from its equivalent value, the osmolar value has been placed in parentheses beneath the equivalent value. The cell water is divided into two columns to illustrate the fact that there must be equal concentrations of anions and cations within it.

Figure 2 represents a glycogen-free liver cell, in which the cell solids are composed completely of protein. In order to maintain osmotic equilibrium with the extracellular fluid, the intracellular water of this cell must contain 320 milliosmols per liter. On the side of the cations, sodium and potassium contribute 187 milliosmols and magnesium 10, making a total of 197 milliosmols. On the side of the anions, bicarbonate accounts for 10 and protein, organic acids, and sulfate account for about 5, raising the total number of milliosmols present to 212. If all of the phosphate that is present in glycogen-free liver were osmotically active in the intracellular water, it would be present at a concentration of 246 milliosmols per kilogram, giving a total intracellular concentration of 458 milliosmols, as opposed to an extracellular osmolarity of 320. Furthermore, assuming bicarbonate to contribute 10 equivalents and protein, sulfate and organic acids 57, phosphate, with a dissociation constant of 1.3, would contribute 320 equivalents, making a total of 387 anions present in association with only 207 cations. A more feasible situation is the one which is represented in Figure 2 in which only 108 milliosmols of phosphate, yielding 140 milliequivalents, are present as acid-soluble phosphate in the intracellular water. This gives the intracellular water a total osmolarity of 320 and a total of 207 anions present with 207 cations. The remainder of the phosphate, some 138 millimols, must exist in the cell in the acid-insoluble fraction, in an osmotically and electrically inactive form, probably bound to protein.

Figure 3 represents a liver cell containing a high concentration of glycogen. Fifty per cent of the dry solids are glycogen. The remainder are protein. The deposition of this glycogen, accompanied by water, has caused the cell to increase 100 per cent in size. Assuming that the concentration of magnesium in the cell water remains constant, the sum of the cations has not changed. However, there has been a marked drop in the concentration of total phosphate. This drop is compatible with dilution of the phosphate present in the glycogen-free cell by the glycogen and water added, for it follows a straight line, as seen in Figure 1. Since the osmolarity of the extracellular fluid does not change in the animal with high liver glycogen (see Figure 1) the osmolarity of the intracellular fluid must also remain 320. The osmolar sum of the cations within the cell is again 197, and the anions bicarbonate, protein, organic acids and sulfate are presumed to contribute 15 more milliosmols, raising the osmolarity of the cell water to 212. If all of the phosphate that is present in a liver containing 50 grams of glycogen per 100 grams of dry solids were osmotically active in the intracellular water, there would be 130 milliosmols of phosphate, yielding a total osmolarity of 342, as opposed to the extracellular osmolarity of 320. However, if we again assume that 108 milliosmols of this phosphate are in solution in the intracellular water, osmotic equality exists between the extracellular and intracellular fluids and if this phosphate has a dissociation constant of 1.3, electroneutrality is achieved between cations and anions.

From these data, it appears that the liver cell containing 50 grams of glycogen per 100 grams dry fat-free solids has only 22 millimols of phosphate remaining in a bound form. These 22 millimols are probably associated with phospholipid and nucleic acids. This is less phosphate than is found in the phospholipids and nucleic acid of normal livers. One must therefore postulate that there is a marked decrease in the phospholipids

and/or nucleoproteins of liver cells during glycogen deposition. The present investigation does not enable us to distinguish whether this is due to glycogen deposition *per se* or whether cortisone administration affects the concentration of these compounds.

Since it appears evident from these experiments that there is no addition of phosphate to the liver during the deposition of glycogen, one is led to deduce that the fall in serum inorganic phosphate observed after the ingestion or infusion of glucose is associated entirely with the peripheral utilization of glucose. This hypothesis has been suggested by other workers, notably Bolliger and Hartman (19) in 1925, and Pollack, Millet, Essex, Mann, and Bollman (20) in 1934. More recently, several clinical observations in support of this concept have been made. In 1952, Groen and his coworkers (21) showed that a drop in serum inorganic phosphate was obtained in a patient with a necrotic liver, but was absent in a patient with pronounced muscular wasting. Observations by Van Bekkum and Querido further substantiate this theory (22). They studied a group of patients with progressive muscular dystrophy and observed that the drop in serum inorganic phosphate following glucose administration was significantly lower than in normal individuals.

SUMMARY

1. As glycogen concentration increases in the liver, the level of total phosphate declines. No extrahepatic phosphate is added to the liver during the deposition of glycogen up to 16 per cent of the liver (by wet weight).

2. The liver contains, at all levels of glycogen studied, more phosphate than can be completely ionized in the intracellular water if osmotic equality with the extracellular fluid and electroneutrality within the cell are to be maintained.

3. Since during glycogen deposition, total phosphate concentrations in liver decline while acid-soluble phosphate concentrations remain constant, there must be a conversion of acid-insoluble phosphate to acid-soluble phosphate during glycogen deposition.

4. These results support the theory that the fall in serum inorganic phosphate associated with the infusion of glucose is a measure of the peripheral utilization of glucose.

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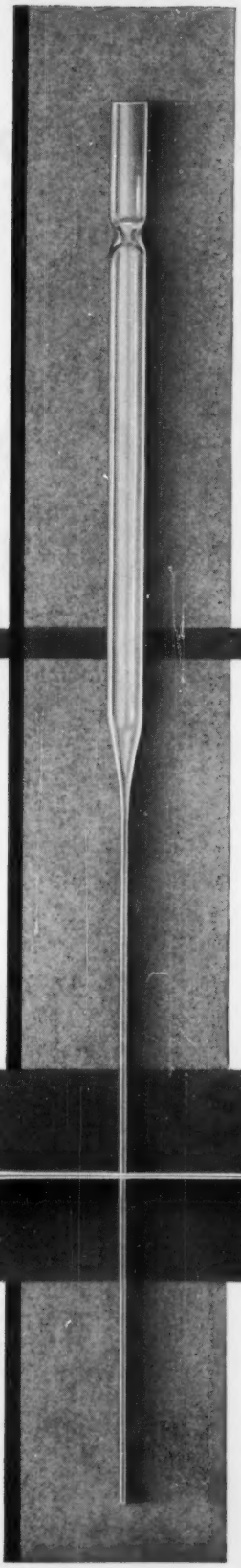

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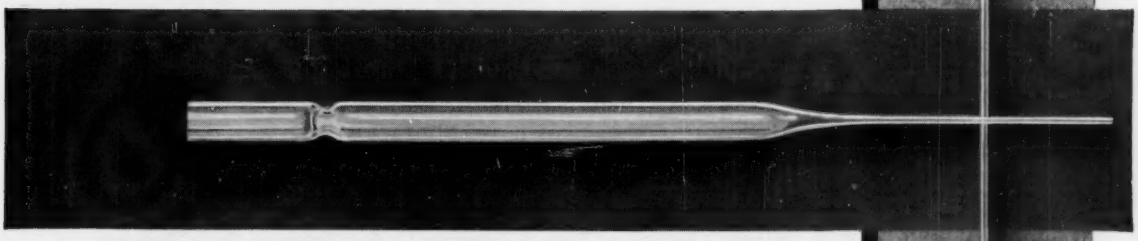

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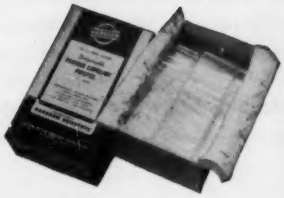

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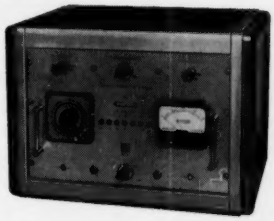

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